Universidade do Minho
Escola de Engenharia

Eva Patricia Paiva Santos Pinho

Development of a New Antimicrobial Material for Wound Dressing

April 2014

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PhD in Biomedical Engineering

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“O que prevemos raramente ocorre; o que menos esperamos geralmente acontece.”

Benjamin Disraeli


ON.2
O NOVO NORTE
PROGRAMA OPERACIONAL
REGIONAL DO NORTE


POPH
PROGRAMA OPERACIONAL POTENCIAL HUMANO


QREN
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DE REFERÊNCIA
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"O mundo inteiro puxa-nos para baixo, mas as mãos de quem gosta de nós atiram-nos para o alto. Sem se cansarem."

Afonso Cruz

(O pintor debaixo do lava-loiças)

Como um alpinista a escalar uma montanha, trouxe comigo uma mochila cheia de auxílio!

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Abstract

Nowadays, wound dressings are complex materials that, behind the mechanical protection, are also capable of interacting with the injury tissue. These complex products improve the healing process by maintaining suitable conditions, allowing skin to establish integrity with appropriate cosmetic results. The incidence of chronic wounds related with diseases, population ageing, and tissue infection caused by multi-resistant strains of bacteria, has been increasing. Thus, the wound dressing research is now focused on the development of new materials with higher specificity in order to improve the quality of patients' life. One of the directions is the use of natural materials for wound dressing's production for the development of a friendlier product.

Hydrogels have been successfully used as wound dressings. They gather favourable properties for wound healing, such as good biocompatibility and ability to maintain the proper environmental humidity, which facilitate the ready removal of the material with minimal pain. However, hydrogels lack the capacity to load and control the release of bioactive molecules. Thus, the incorporation of cyclodextrins on the hydrogels allowed the improvement of the polymeric network ability to protect and modulate the release of bioactive molecules. Additionally, cyclodextrins are capable of complex with a wide range of molecules, due to their micro-heterogeneous environment (hydrophilic outside and hydrophobic cavity). Moreover, plants have been used as source for new therapeutic agents able to enhance the healing process. Polyphenolics, one of the groups of bioactive molecules, has been described as anti-oxidant, anticarcinogenic, anti-inflammatory and antimicrobial. However, their application by the pharmaceutical industry is limited by polyphenolics lower solubility and stability to environmental stress. Thus, cyclodextrins has been proposed as viable carrier for the maintenance of bioactive molecules' structural integrity and bioactivity.

Therefore, the present thesis reports the work developed to obtain a wound dressing, using natural compounds, capable of preventing infection of the injury tissues.

The first step was to select phenolic compounds from natural extracts obtained from Portuguese wild plants. Gallic and caffeic acid revelled to be the most efficient antibacterial agents against bacteria commonly isolated from skin and soft tissue infections (*Klebsiella pneumoniae, Staphylococcus epidermidis and Staphylococcus aureus*). Besides, gallic acid has shown very low cytotoxicity, for concentrations lower than 0.01 $mg.mL^{-1}$.

In order to enhance gallic and caffeic acids solubility and stability, their encapsulation by cyclodextrins was evaluated. The inclusion complexes obtained were stable and capable of destroying the bacteria used. Although, gallic and caffeic acid had similar molecular structure, the cyclodextrins encapsulation behaviour was different. In the case of gallic acid, the hydroxypropyl-β-cyclodextrin formed a more stable inclusion complex at pH 3 (when compared with β-cyclodextrin and methyl-β-cyclodextrin) as well as presented a better antibacterial activity. Moreover, gallic acid carboxylic group was positioned towards the smaller cyclodextrins 'cavity and hydroxyl groups were placed near the wider opening. The gallic acid neutral form, positioned inside the cyclodextrins, preserved gallic acid antibacterial activity. Otherwise, the caffeic acid carboxylic group was project to the water phase and the aromatic part inside the cyclodextrins cavity. Additionally, the native cyclodextrin was better for the encapsulation of this phenolic at pH 5 and it antibacterial activity was enhanced by the inclusion complex formation.

Hence, the incorporation of gallic and caffeic acid in a polymeric network was, also, studied. A hydrogel based on cyclodextrins and hydroxypropyl methylcellulose, using 1,4-butanediol diglycidyl ether as cross-linking agent, was synthetized. Only the β-cyclodextrin and hydroxypropyl-β-cyclodextrin permitted the formation of hydrogels with good mechanical properties. Both networks behaved with superabsorbent materials and were capable of loading and release gallic and caffeic acid by a control mechanism. The differences between the 2 phenolic acids found in the study of the inclusion complexes were similar to the load hydrogels. The gel with hydroxypropyl-β-cyclodextrin had better properties regarding the gallic acid and the gel with β-cyclodextrin was more suitable in the caffeic acid case. Thus, cyclodextrins were the major mechanism involved on the load and release of the gallic and caffeic acid. Furthermore, the phenolic acids antibacterial activity and effect on the fibroblasts proliferation was maintained after their incorporation on the hydrogels.

In conclusion, the work developed proved that natural antibacterial agents can be used to control the growth of the most common bacteria isolated from infected wounds, and their incorporation in cyclodextrins-based hydrogels preserved the selected phenolics biological activities. Thus, the hydrogels synthesised could be useful as natural antibacterial agent's delivery systems device for the treatment of wound infections.

Sumário

Atualmente, os pensos utilizados no tratamento de feridas são materiais complexos que oferecem proteção mecânica e promovem cicatrização, mantendo as condições mais favoráveis para o restabelecimento da integridade da pele. A incidência de feridas crónicas como consequência de doenças, do envelhecimento da população e de infeções provocadas por bactérias multirresistentes, tem aumentado nos últimos anos. Esta evolução obrigou a indústria a direcionar os seus esforços para o desenvolvimento de materiais com maior especificidade, contribuindo para melhorar a qualidade de vida dos pacientes.

Os hidrogéis reúnem propriedades que favorecem a cicatrização de feridas, nomeadamente biocompatibilidade e capacidade para manter a humidade, o que facilita a remoção dos dispositivos com reduzido trauma ou dor para o paciente. Por isso mesmo, têm sido aplicados com sucesso no tratamento de feridas crónicas. Contudo, a sua capacidade para adsorver e libertar fármacos de um modo controlado é reduzida. Assim, a incorporação de ciclodextrinas em matrizes poliméricas tem sido investigada de modo a melhorar a capacidade dos hidrogéis para proteger e modular a libertação de biomoléculas. As ciclodextrinas apresentam, ainda, a vantagem de conseguirem encapsular uma grande variedade de moléculas, pois possuem uma superfície externa hidrofílica e uma cavidade hidrofóbica.

A procura de novos agentes antimicrobianos tem crescido nos últimos anos como consequência do decréscimo de eficiência dos antibióticos contra bactérias multirresistentes. As plantas possuem uma gama alargada de moléculas, por exemplo polifenólicos, capazes de promoverem a cicatrização e prevenirem infeções. Os polifenólicos têm sido descritos como antioxidante, anti-carcinogénico, anti-inflamatório e antimicrobiano. No entanto, a sua utilização pela indústria farmacêutica é limitada, devido à sua baixa solubilidade e estabilidade. Assim sendo, a encapsulação de compostos polifenólicos com ciclodextrinas tem sido proposta como opção viável, pois a sua integridade estrutural e a bioatividade são preservadas após inclusão.

A presente tese resume o trabalho desenvolvido na conceção de um penso para o tratamento de feridas com compostos naturais, capaz de melhorar a cicatrização e de prevenir infeções.

Inicialmente, procedeu-se à seleção de compostos fenólicos obtidos a partir de extratos de plantas do Nordeste português. Os ácidos gálico e cafeico foram os compostos polifenólicos que apresentaram maior eficiência no controle da proliferação de bactérias, normalmente isoladas de

feridas infetadas (*Klebsiella pneumoniae, Staphylococcus epidermidis and Staphylococcus aureus*). Além do mais, o ácido gálico apresentou reduzida citotoxicidade para concentrações abaixo de 0.01 $mg.mL^{-1}$.

No sentido de melhorar as propriedades de estabilidade e solubilidade dos ácidos gálico e cafeico procedeu-se ao estudo da sua encapsulação com ciclodextrinas. Os complexos de inclusão obtidos demostraram ser estáveis e capazes de manter a atividade antibacteriana dos fenólicos. Apesar dos ácidos gálico e cafeico apresentarem uma estrutura molecular semelhante, o comportamento de encapsulação com ciclodextrinas divergiu. No caso do ácido gálico, a hidroxipropil-β-ciclodextrina permitiu a formação de complexos mais estáveis a pH 3 e com melhor atividade antibacteriana. E, supõe-se que o ácido gálico encapsulado apresente o grupo carboxílico orientado para a abertura mais pequena da ciclodextrina e os grupos hidroxilos voltados para o interior da cavidade. Pelo contrário, o grupo carboxílico do ácido cafeico estará projetado para fora da ciclodextrina e a parte aromática para o interior da cavidade. Além disso, a β-ciclodextrina foi a mais eficiente na complexação do ácido cafeico a pH 5 e observou-se um reforço das propriedades antibacterianas deste fenólico depois da inclusão.

Após a confirmação da complexação dos ácido fenólicos pelas ciclodextrinas com preservação das propriedades antibacterianas, estudou-se a incorporação dos ácidos gálico e cafeico num hidrogel. Para tal, produziu-se uma matriz polimérica utilizando ciclodextrinas e hidroxipropil metil celulose como monómeros e 1,4-butanediol diglicidil éter como agente de reticulação. A formação de hidrogéis com propriedades mecânicas aceitáveis só foi confirmada com a utilização da β-ciclodextrina e da hidroxipropil-β-ciclodextrina. Em ambos os casos obteve-se materiais superabsorventes, em que as ciclodextrinas desempenharam um papel crucial na capacidade de modelar a adsorção e a libertação dos ácidos fenólicos. Além disso, a atividade antibacteriana e o efeito sobre a proliferação de fibroblastos manteve-se após incorporação dos ácidos fenólicos nos hidrogéis.

Em conclusão, o trabalho desenvolvido demostrou ser possível utilizar ácidos fenólicos de origem natural para controlar o crescimento das bactérias normalmente isoladas de feridas infetadas. Comprovou-se, ainda, a possibilidade de incorporação destes compostos antimicrobianos em hidrogéis com ciclodextrinas mantendo as suas propriedades biológicas. Assim sendo, os hidrogéis antibacterianos sintetizados poderão ser úteis na prevenção e tratamento de infeções em feridas.

Scope

Wounds result from skin injury caused by mechanical, thermal, chemical, electric or by the presence of an underlying medical or physiological disorder. Normally, the human body is capable of restore the skin integrity with minimal scar, by a complex and interactive cascade of events. However, in some situations the normal healing process is interrupted as result of physical factors like age, nutritional status or local factors such as infections, tissue ischaemia, haematomas, foreign bodies or mechanical pressure. In these situations, medical treatment is necessary. Additionally, wounds related to pathologies diseases such as diabetes mellitus, chronic venous, arterial insufficiency, and immunological or dermatological diseases have increased, in the last years. This demand for an effective management of the different types of wounds induced the commercialization of a wide range of wound dressings.

Wound dressings evolved from simple plan textile strips to engineered composite materials with different layers and, even, with medical substances inside. At this point, the wound dressings research aims to develop a wound dressing capable of improving the healing process by maintaining the favourable conditions for the re-establishment of the skin integrity to achieve the best function and cosmetic results. Thus, wound dressings have been used as platforms for the sustained delivery of therapeutic agents for a desired period of time. This feature has been particularly important regarding the control of wounds infections. In fact, the deposition and proliferation of microorganisms, such as *Staphylococcus aureus, Staphylococcus epidermidis, Escherichia coli, Pseudomonas aeruginosa* and *Candida species,* on the injury tissues lead to wound infection, inducing tissue inflammation and triggering the immune response. Therefore, wound infections can prolong or impair the wound healing process, resulting in tissue morbidity and, ultimately, on sepsis and dead.

The population ageing associated with the emergence of multi-resistant strains of bacteria, resultant from to the widespread use of broad-spectrum antibiotics, has been challenging the industry to find new sources of antibacterial agents, as well as new wound dressings more specific for each diseases. So, the use of natural materials for wound dressings production has gained prominence.

Aim

Considering the facts exposed, the prime goal of the present thesis was to develop a wound dressing with the ability to promote the healing process and, also, capable of preventing the infection of the injury tissue. Thus, the first aim was the characterization and selection of antibacterial agents obtained from Northeast Portuguese plants for the substitution of large-spectrum antibiotics, normally, used for the treatment of infected wounds. Then, hydrogels based on natural monomers were developed and characterized in order to be applied as platforms for the delivery of antibacterial agents, for wound dressing proposes. In order to achieve this last goal, the encapsulation of natural agents by cyclodextrins was optimized.

Structure

The present dissertation gathers the scientific work developed to accomplish the goals proposed above. It was structured in five chapters, according with the following organization.

Chapter 1 overviews the state of the art regarding the natural antibacterial agents obtained from plants, with specially emphasis on polyphenolic compounds, as well as their encapsulation by cyclodextrins for improvement of polyphenols stabilization and solubilisation, to be then incorporated in hydrogels for wound care.

Chapter 2 is focused on the antibacterial activity evaluation of phenolic extracts obtained from wild plants collected on Northeast Portugal region. The potential antibacterial activity of phenolic compounds obtained from those extracts is, also, described in this chapter.

Chapter 3 gathers 2 subchapters concerning the evaluation of cyclodextrins encapsulation on the gallic acid (3.1) or caffeic acid (3.2) antibacterial activity, respectively. Physiochemical and biological characterization of the complexes was used for the evaluation.

Chapters 4 report the synthesis of cyclodextrin-based hydrogels and their physicochemical characterization. The effect of hydrogel functionalization by the gallic acid (chapter 4.1) or caffeic acid (chapter 4.2) incorporation is, also, described and characterized.

Chapter 5 closes the thesis by connecting the most significant conclusions obtained in the present work and pointing out relevant paths for future research.

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List of Abbreviations, Acronyms and Symbols

Aa	*Asparagus acutifolius*
ATCC	American type culture collection
Bd	*Bryonia dioica*
BDGE	1,4-Butanediol diglycidyl ether
CD	Cyclodextrin
CFUs	Colony forming units
CIMO	Mountain research centre
Cl	*Cistus ladanifer*
CLSI	Clinical and Laboratory Standards Institute
Cm	*Cytisus multiflorus*
CMEβCD	*O*-carboxymethyl-*O*- ethyl- beta–cyclodextrin
CMβCD	*O*-carboxymethyl- beta–cyclodextrin
Cs	*Castanea sativa*
DEβCD	2,6-diethy-beta–cyclodextrin
DHPβCD	2,3-dihydroxypropyl-β-cyclodextrin
DMAβCD	Cetylated-2,6-dimethyl-beta–cyclodextrin
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DMβCD	2,6-dimethyl-beta–cyclodextrin
DSC	Differential scanning calorimetry
ECM	Extracellular matrice
EGDE	Ethylene glycol diglycidyl ether
EMA	Ethyl methacrylate
EPH	Epichlorohydrin
ESB	Polytechnic Institute of Bragança
EUCAST	European Committee on Antimicrobial Susceptibility Testing
FDA	Food and Drug Administration
FTIR	Fourier transform infrared spectroscopy
Fu	*Filipendula ulmaria*
G_1βCD	Glycosyl- beta–cyclodextrin
G_2βCD	Maltosyl– beta–cyclodextrin
gel-HPβ	HPβCD-co-HPMC
gel-β	βCD-co-HPMC
GUGβCD	Glucuronyl-glucosyl- beta–cyclodextrin
HA	Hyaluronic acid
HCA	Hydrocortisone acetate
HEMA	Hydroxyethyl methacrylate
HEβCD	Hydroxyethyl- beta–cyclodextrin

HPMA	Hydroxypropyl methacrylate
HPMC	Hydroxypropyl methylcellulose
HPαCD	2- hydroxypropyl-alpha–cyclodextrin
HPβCD	2- hydroxypropyl- beta–cyclodextrin
HPγCD	2- hydroxypropyl- gamma–cyclodextrin
HSDB	Hazardous substances data bank
HTAβCD	Hydroxy trimethyl ammonium propyl-beta–cyclodextrin
IC	Inclusion complex
ISO	International standard organization
K	Stability constant
MAH	(Meth)acrylated hyaluronic acid
MBC	Minimal bactericide concentration
MeOH	Methanol
MHB	Mueller–Hinton broth
MIC	Minimal inhibitory concentration
MTS	[3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium]
MβCD	Methyl- beta–cyclodextrin
NCCLS	National Committee for Clinical Laboratory Standards
NMR	Nuclear magnetic resonance
O-CD	Cyclodextrin-oligomers
OH	Hydroxyl groups
PAA	Poly(acrylic acid)
PAAm	Polyacrylamide
PBO	Poly(butylene oxide)
PBS	Phosphate buffer solution
PCL	Polycaprolactone
PEG	Poly(ethylene glycol)
PEG-THF	Poly(ethyleneglycol mono-tetrahydrofurfuryl ether
PEI	Polyethyleneimine
PEO	Poly(ethylene oxide)
PF	Propylene fumarate
PGEMA	Poly(glucosylethyl methacrylate)
PHB	Poly(hydroxy butyrate)
PHEMA	Polyhydroxybutyrate
PLA	Poly(lactic acid)
PLGA	Poly(lactic-co-glycolic acid)
PMMA	Poly(methyl methacrylate).
PNIPAAm	Poly(n-isopropylacrylamide)
PNVP	Poly (N-vinylpyrrolidone)
PPO	Poly(propylene oxide)
PVA	Poly(vinyl alcohol)
PVAc	Poly(vinyl acetate)
Q	Degree of swelling

Rm	*Rosa micrantha*
RMβCD	Randomly methylated-beta–cyclodextrin
ROS	Reactive oxygen species
Rpm	Rotations per minute
SBEβCD	Sulphate and sulfobutyl ether beta–cyclodextrin
SEM	Scanning electron microscope
SF	Silk fibroin
SM	Supramolecular structures
Sn	*Sambucus nigra*
SSS	Synthetic sweat solution
TaβCD	*Per-o*-acetyl- beta–cyclodextrin
TEβCD	*Per-o*-ethyl-beta–cyclodextrin
TMβCD	2,3,6,-trimethyl- beta–cyclodextrin
TSA	Tryptic soy agar
TSB	Tryptic soy broth
TVβCD	*Per-o*-valeryl- beta–cyclodextrin
UV	Ultraviolet
UV-Vis	Ultraviolet- visible
W	Weight of fully swollen hydrogel in water
W_∞	Amount of water at equilibrium
W_0	Weight after the dry process
W_t	Amount of water absorbed at time t
αCD	Alpha-cyclodextrin
βCD	Beta-cyclodextrin
γCD	Gama-cyclodextrin
ΔG	Gibbs free energy
ΔH	Enthalpy
ΔS	Entropy
λ_{max}	Maximum wavelength

List of Publications

The work developed during the four years of the PhD program resulted in several publications in peer-reviewed international journals, as well as abstracts in proceedings of international conferences. These publications are compiled below.

Papers in peer-reviewed international journals

Pinho E, Grootveld M, Soares GMB, Henriques M: **Cyclodextrins as encapsulation agents for plant bioactive compounds**, *Carbohydrate Polymers* 2013, **101**(30):121-135. doi: 10.1016/j.carbpol.2013.08.078

Pinho E, Grootveld M, Soares GMB, Henriques M: **Cyclodextrin-based hydrogels toward improved wound dressings**, *Critical Reviews in Biotechnology* 2013, **8551**:1-10. doi:10.3109/07388551.2013.794413.

Pinho E, Ferreira I, Barros L, Carvalho A, Soares GMB, Henriques M: **Antibacterial potential of North-eastern Portugal wild plant extracts and respective phenolic compounds**. *BioMed Research International* (accepted).

Pinho E, Soares GMB, Henriques M: **Cyclodextrin modulation of gallic acid in vitro antibacterial activity**. Journal of *Inclusion Phenomena and Macrocyclic Chemistry* (Submitted).

Pinho E, Soares GMB, Henriques M: **Evaluation of antibacterial activity of caffeic acid encapsulated by β-Cyclodextrins.** *International Journal of Biological Macromolecules* (Submitted).

Pinho E, Henriques M, Soares GMB: **Cyclodextrin/cellulose hydrogel with gallic acid to prevent wound infection**. *Cellulose* (Submitted).

Pinho E, Henriques M, Soares GMB: **Caffeic acid loading wound dressing: Physicochemical and Biological characterization**. *Future Microbiology* (Submitted).

Abstracts and proceedings in international conferences

Pinh, Eva, Soares G.M.B, Henriques M. **Gallic acid and cyclodextrins: inclusion complexes and antimicrobial activity**, *BioMicroWorld 2013 - V International Conference Environmental, Industrial and Applied Microbiology*, 2013.

Pinho Eva, Grootveld Martin, Soares G.M.B, Henriques M. **Assessment of antimicrobial activity of textiles for wound dressing: methodology optimization**, *TRS 2012 – The 41th Textile Research Symposium*, 2012.

Pinho Eva, Barros Lillian, Soares G.M.B, Ferreira Isabel C.F.R, Henriques M. **Screening of the antibacterial activity of phenolic extracts from Portuguese north-eastern plants**, *ICAR 2012 - II International Conference on Antimicrobial Research*, 2012.

Pinho Eva, Grootveld Martin, Soares G.M.B, Henriques M. **Assessment of antimicrobial activity of textiles for wound dressing: methodology optimization***, MicroBiotec'11*, 2011.

Chapter 1

General Introduction

THE PRESENT CHAPTER WAS ADAPTED FROM THE FOLLOWING REVIEW PAPERS

Pinho E, Grootveld M, Soares GMB, Henriques M. Cyclodextrin-based hydrogels toward improved wound dressings, Critical Reviews in Biotechnology, 8551, 1-10,2013 doi:10.3109/07388551.2013.794413

Pinho E, Grootveld M, Soares GMB, Henriques M. Cyclodextrins as encapsulation agents for plant bioactive compounds, Carbohydrate Polymers, 101(30), 121-135, 2013. doi: 10.1016/j.carbpol.2013.08.078

Abstract

Optimal wound dressings should be capable of mechanical wound protection combined with the ability to facilitate the healing process, via maintenance of suitable environmental conditions and the controlled delivery of bioactive molecules.

Hydrogels present suitable properties for wound dressing applications such as good biocompatibility, together with a high water content, the latter of which is important for the maintenance of a moist environment and ready removal from the wound with a minimal level of associated pain. However, they have poor properties as drug-delivery systems. Thus, the conjugation with cyclodextrins may allow the achievement of an optimal wound dressing material, since the hydrogel component will maintain the moist environment required for the healing process, and the cyclodextrin moiety has the ability to protect and modulate the release of bioactive molecules.

Cyclodextrins are cyclic oligosaccharides arising from the starch degradation, which can be a viable option as encapsulation vehicle. Cyclodextrins are inexpensive, friendly to humans, and also capable of improving the biological, chemical and physical properties of bioactive molecules.

Plants possess a wide range of molecules capable of improve healing: fibre, vitamins, phytosterols, and further sulphur-containing compounds, carotenoids, organic acid anions and polyphenolics. However, they require an adequate level of protection, from the environmental conditions, to prevent losing their structural integrity and bioactivity.

In the present chapter, cyclodextrins were analysed simultaneous as monomers for hydrogels synthesis and as encapsulation agent for antibacterial agents.

Keywords: Bioavailability, cyclodextrin; drug delivery, hydrogel, infection, inclusion complex; polyphenolic, supramolecular structures, wound dressing.

Introduction

The skin is the largest human organ, and probably the most heterogeneous, reaching 10% of the total body mass [1]. The main function of the skin is to act as a protective barrier against the environment, and beyond this physical protection function, skin is also responsible for sensory detection, thermoregulation, fluid homeostasis, immune surveillance, and self-healing [2]. Normally, the human body is able to restore skin integrity after injury, with a minimal scar, via a complex and interactive process. However, this healing process can be interrupted by a series of physical factors such as age, nutritional status, or local factors, like infections, tissue ischaemia, haematomas, foreign bodies or mechanical pressure. In these situations, medical treatment is necessary [3, 4]. Indeed, in the past few years the number of wounds related to diseases such as diabetes mellitus, chronic venous disease, arterial insufficiency, and immunological or dermatological illnesses have increased. Therefore, in order to improve the life quality of those affected, a range of wound-care products have been developed [5–8].

Wound dressings cover the wound, providing physical protection against microorganism deposition, wound dehydration, and external injuries [9, 10]. Moreover, they can interact with the wound and accelerate the healing process, via the release of bioactive molecules, and/or by maintaining the moist environment required for effective wound healing [11]. New formulations of composite systems containing synthetic and/or biological agents, such as gauzes, foams, films, hydrocolloids and hydrogels, have been developed to maintain the favourable environmental conditions, and/or to deliver bioactive compounds through the skin and, hence, enhance the healing process [6].

Hydrogels, composed of polymeric networks with a high water content and biocompatibility status, can be used as wound-care coverings, drug delivery systems, dental materials, implants, injectable polymeric systems, ophthalmic application systems and hybrid-type organs [4]. Additionally, the presence of cyclodextrins (CD) on hydrogels as drug carriers improve the properties of the polymeric networks and give rise to an enhanced level of wound healing [12–14].

CDs can serve as vehicles for the protection of bioactive molecules due to their ability to encapsulate molecules, a process involving the formation of inclusion complexes (IC). Indeed, CDs have the capacity to modify the guest molecule's characteristics. For example, they enhance

the solubility of lipophilic 'guests', stabilise the 'guest' against derivatizing agents (such as oxygen, visible or ultra-violet light, and heat), control volatility and sublimation properties, allow the physical isolation of incompatible compounds (via chromatographic separation), permit taste modification by masking potentially adverse flavours, control odours and the release of such encapsulated compounds. Furthermore, CDs are now readily available, and their price and production costs have declined in recent years [15–20].

Plants are, virtually, inexhaustible sources of biologically-active compounds, which are their defence mechanisms against microorganism, insects and herbivores. Plants bioactive compounds have been, widely used by the food, cosmetic and pharmaceutical industries [21–24]. In the pharmaceutical field, the utilization of natural compounds instead of synthetic ones offers many advantages, such as high levels of biocompatibility, low toxicity and good availability. Plants' bioactive molecules include polyphenolics, alkaloids and polysaccharides, and they, all, have well-documented pharmacological properties. However, polyphenolics are currently one of the major group of interest in view of their anti-inflammatory, anti-microbial and anti-oxidant properties, as well their availability in the human diet [21–24].

In order to have biological activity, molecules need to be capable of reaching the action site without losing integrity and to be able to cross the lipophilic membrane. Plants bioactive compounds have restrict application as pharmaceutical products since they have limited water solubility, poor bioavailability, and can be easily modified by environmental factors, such as temperature, pH and light. Therefore, in order to preserve their structural integrity, these kind of molecules need to be protected by a finishing formulation with the capacity to deliver them to the physiological targets, without losing any bioactivity [25, 26].

The present chapter gathers the state of the art on two main applications of CDs for the development of optimal wound dressing. An overview of the CDs as monomers for synthesis of hydrogels, improving their drug delivery properties is provided. Additionally, a synthesis of the complexes between CDs and plant polyphenolics, with pharmaceutical applications, is also discussed.

Cyclodextrins

Cyclodextrins (CDs) are a group of structurally-related cyclic oligosaccharides described for the first time by Schardinger [17, 27]. They are produced by the bacterial enzymatic degradation of starch [15, 16, 18, 27]. The naturally-occurring CDs (Fig 1.1) are αCD with 6 units, βCD with 7 units, and γCD with 8 units [17, 28, 29]. These 3 CDs have the same cavity height; however, their cavity volume and diameter varies, which determines the classes of molecules ('guest' molecules) that will fit better in each CD cavity. βCD is more accessible, with a lower cost price, and is, also, the most commonly employed one for commercial proposes [15].

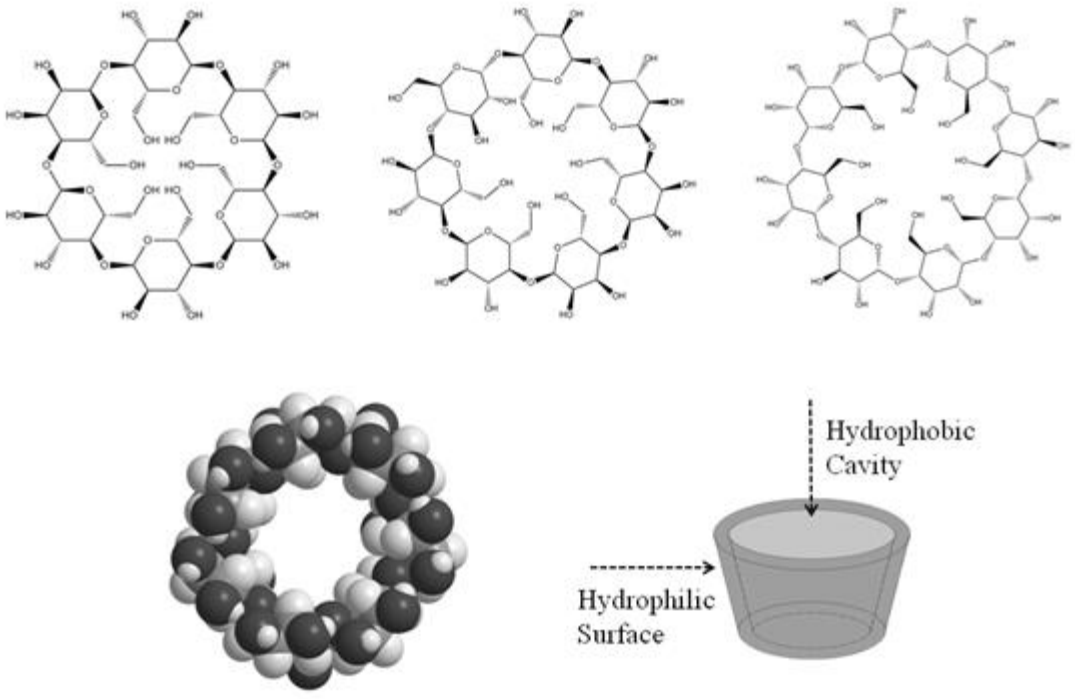


Fig 1.1 Schematic representation of αCD, βCD and γCD (left to right) and schematic representation of the CD truncate aspect (Chem3D Pro 12.0 software).

CDs have the shape of a truncated cone (Fig 1.1), mainly in view of the chair conformation of the glucopyranoside units [30, 31]. Since their hydroxyl groups (OH) are oriented to the outer

molecular surface, CDs have a micro-heterogeneous environment, the outside having hydrophilic characteristics and the inner cavity being a hydrophobic environment [16, 18]. Therefore, CDs are able to accommodate poorly water-soluble molecules (such as polyphenolics), improving molecules' solubility or even polymers forming IC in aqueous solutions [17, 18, 27, 31, 32]. In addition to the solubilisation improvement, CDs protect bioactive molecules from side-effects caused by environmental conditions (temperature, pH, light) and, hence enhance their shelf-life and reduce the concentrations of the agent required to achieve a biological effect [26, 33]. Moreover, CDs can modify the behaviour of the encapsulated molecule, specifically by modulate the delivery rate (i.e. retarding or prolonging it), which renders the drug formulation with CDs 'non-bioequivalent' to formulations without CDs [29, 34]. Other advantage of the CDs application as carrier is CD' ability to eliminate irritant or toxicological effects of the active agent, by replace some excipients, such as pH regulators, solubilising agents or organic solvents [29, 35–37].

Cyclodextrins' Derivatives

In the last years, physicochemical properties and, consequently, the inclusion capacity of the natives CD have been improved by chemical modification of their OH [28]. The CD derivatives may help to control the chemical activity of the 'guest' molecule, and chemical modifications of CDs enlarge the applications of such molecules. For example, functional groups that act on molecular recognition can be added to CDs, enabling their use in enzyme mimetization, targeted drug delivery, or analytical chemistry applications [15, 19].

CD derivatives can be manufactured by aminations, esterifications or etherifications of the primary or secondary OH from naturally occurring CDs [27]. Each CDs' glucopyranose unit has 3 reactive OH with different ratio of reactivity and function, in the case of βCD it is possible to change 21 OH by chemical or enzymatic reaction [31]. The βCD derivatives (Table 1.1) are, normally, distributed based in their interaction with the water molecules, i.e, hydrophilic, hydrophobic or ionisable derivatives. The first group (hydrophilic) has better solubility in water and are suitable for IC formation with poor water soluble "guest" molecules. The DMβCD, TMβCD, hydroxyalkylated CDs, such as HPβCD, and branched CDs, like G_1βCD, are some examples of hydrophilic CD derivatives. The hydrophobic derivatives, for example DeβCD, are capable of decrease and modulate the release rate of water soluble molecules. The ionisable CDs CMβCD, CMEβCD, and SBEβCD, can enhance the dissolution rate, the inclusion capacity and,

also, the decrease of the side effects of some molecules [27, 28]. The HPβCD and the SBEβCD are the most used derivatives on the pharmaceutical industry, their low toxicity and high solubility make them suitable for oral and parental application (Table 1.1) [27, 37, 38]

Table 1.1 Common βCD derivatives

CD'Derivative	Characteristic
Hydrophilic derivatives	
MβCD	Soluble in cold water and in organic solvents,
DMβCD	surface active, hemolytic
TMβCD	
DMAβCD	Soluble in water, low hemolytic
Hydroxyalkylated βCD	
2HEβCD	Amorphous mixture with different degrees of substitution,
2HPβCD	highly water-soluble (50%), low toxicity
3HPβCD	
3HPβCD	
2,3-DHPβCD	
Branched βCD	
G_1βCD	Highly water-soluble (50%), low toxicity
G_2βCD	
GUGβCD	
Hydrophobic derivatives	
AlkylatedβCD	
DEβCD	Poorly water-soluble, soluble in organic solvents, surface-active
TEβCD	
AcylatedβCD (C2—C18)	
TAβCD	Poorly water-soluble, soluble in organic solvents
TVβCD	Film formation
Ionaizable derivatives	
AnionicβCD	p*K*a=3 to 4, soluble at pH 4
CMEβCD	

Inclusion Complex Formation Process

There are several methods for the formation of ICs between CDs and bioactive molecules, and the selection of the process is clearly based on the properties of the guest molecule, the facilities

available and the cost involved [36]. The most common methods are neutralization, slurry, solution, co-precipitation, kneading, and grinding method [39].

The process of inclusion of the 'guest' into the CD occurs at the supramolecular level, and hence there is a substitution of enthalpy-rich water molecules from the central cavity, by the lipophilic 'guest' or moiety, no covalent bonds are broken or formed between the IC compounds [18, 36]. The IC is maintained via hydrophobic forces and van der Waals interactions, and, also, by other factors like the release of ring strain, modifications in solvent surface, tensions and hydrogen-bonds which render the IC complex more energetically-stable [15, 18]. IC generation represents a three-dimensional fit between the CD and the 'guest' molecule, and on the specific local interactions between the CDs' surface groups and the guest molecule [17]. The inclusion process is dynamic and the 'guest' molecule-CD interactions are required to reach equilibrium in order to stabilize the IC [16, 40]. The IC is very stable, and possesses a long shelf-life at ambient temperature and under dry conditions. However, it can be disrupted by increases in temperature, or by exposure of the complex to water, which can replace the 'guest' molecule within the CD cavity [16]. However, this may be helpful when the goal is to achieve a controlled release of the 'guest', for example a drug.

The CD encapsulation of the bioactive molecule induces alterations on the physicochemical characteristics of both agents. Therefore, it is possible to assess the stoichiometry of the complexes and their stability constant (K) by analysing the modifications on the solubility, chemical reactivity and stability, UV-Vis absorbency, drug retention and permeability [36, 41, 42]. The stoichiometry of the IC represents the number of molecules that interact with the CD, in most part of the cases the 1:1 IC is observed, however the same CD can interact with 2 or more molecules (1:2) or one guest can complex with more than one CD (2:1), not so frequent. The variable, K, also known as equilibrium constant or binding constant, needs to be determined by experimental methods and is a parameter that represents the thermodynamic equilibrium between the free and the complexes molecules [43]. Moreover, thermodynamic parameters, such as enthalpy (ΔH), entropy (ΔS) and free Gibbs energy (ΔG), must be, also, considered as important parameters on the evaluation of the complexation process, since the temperature influences the selectivity of the binding between CD and the bioactive molecule [41, 44].

Therefore, in order to assess the K value and the stoichiometry of the complex, evaluation of the IC concentrations and the equilibrium concentrations of the CD and the bioactive molecule needs

to be assessed based on experimental methods. Continuous variation, slope ratio and mole ratio are some of the methods used, and the parameters are measured based on alterations on one or more physicochemical properties of the guest, for example UV-Vis absorbency spectrum or NMR (nuclear magnetic resonance) [42, 44].

Applications

The main interest in CDs lies in their ability to encapsulate 'guest' molecules and, hence, modify their physiochemical characteristics [15, 16]. In fact, CDs can enhance the solubility of lipophilic 'guests', stabilise the 'guest' molecule against potentially damaging agents (such as oxygen, visible or ultra-violet light, and heat), control their volatilities and sublimation properties, allow the physical isolation of incompatible compounds (via chromatographic separation), permit taste modifications by 'masking' flavours, control odours, and attenuate the release of bioactive compounds [17–19].

Besides the improvement of some characteristics of the 'guest' molecule, ICs have low cost and good availability, which make them useful for pharmaceutical applications, analytical sciences, separation processes, and catalysis. They are also of much value to the cosmetic, textile, food and packaging industries [17, 18, 20].

The food industry has used CDs to form ICs with lipids, flavours and colourings. They are very helpful for flavour protection and delivery, and also have the ability to remove some unhealthy compounds (e.g. cholesterol), and, also, improve the textural properties of some foods [27].

CDs are widely used in cosmetic, personal care products, and toiletries, such as toothpastes, skin creams, softeners and tissues. Additionally, antimicrobial agents can be added to toothpastes as ICs [15, 17, 27]. Moreover, ICs are used as chemical stabilisers to prolong the action of active compounds, to decrease local irritation, or, alternatively, to mask unpleasant odours [45]. CDs are used to improve perfume fragrance, enhance the effect of room fresheners by suppressing the volatilities of their odour molecules, and also to control the release of fragrances in detergent formulations.

In the pharmaceutical industry, CDs are used as drug carriers to enhance the solubility, stability and bioavailability of the bioactive molecules [46]. They have a high level of biocompatibility and are approved by FDA (Food and Drug Administration), thus CDs are friendly to humans, indeed

they may only be harmful at extremely high concentrations [16, 28, 42, 47]. CDs are capable of encapsulating a wide range of molecules such as straight or branched aliphatic chains, aldehydes, ketones, alcohols, organic acids, fatty acids, aromatics, gases, and polar compounds like halogens, oxyacids and amines [15].

The pharmaceutical industry possesses a wide range of products with CDs, where they are used as active 'carriers'. Usually, the drugs involved must be able to reach the cellular membrane without a corresponding loss of integrity, and also be hydrophobic enough to cross it. Therefore, CDs can be used to facilitate drug delivery processes by the maintenance of hydrophobic drugs in solution, and deliver them into cell membranes, enhancing their level of penetration via substitution of the drug by blood serum or tissue biomolecules in the cavity [15, 28, 48]. Moreover, CDs are used as solubilizers and stabilisers, and are able to reduce local irritation, a phenomenon attributable to their high level of biocompatibility [15, 18, 27, 28, 42, 49]. Moreover, CDs have been also applied as monomers for hydrogels wound dressings as drug delivery devices [15].

Hydrogels

Hydrogels are tri-dimensional networks of hydrophilic polymers [50], the network confers an insoluble behaviour to the polymeric system and allow the hydrogels to absorb from 10-20% (an arbitrary lower limit) to up to thousands of times their equivalent weight in water, until the process reaches an equilibrium state. Therefore, hydrogels provide the moist environment required for an optimal wound healing process [4, 51, 52]. They are mainly employed to dry-to-moderately draining-wounds, to promote autolytic debridement in necrotic wounds and in granulating wounds.

The characteristics of hydrogels critically depend on the polymers employed and on their interactions within the network. Hydrogels are known as either permanent or chemical if their network is covalently cross-linked [51–53] or physical/reversible if the network is sustained by molecular entanglements and/or secondary attractions, including electrostatic interactions, hydrogen-bonding or hydrophobic forces (Van der Waals) (Fig 1.2). The reversible character of these hydrogels arises from the disruption of the above network interactions via modifications in physical conditions such as ionic strength, pH, temperature, stress or, alternatively, the addition of specific solutes [51, 52, 54, 55].

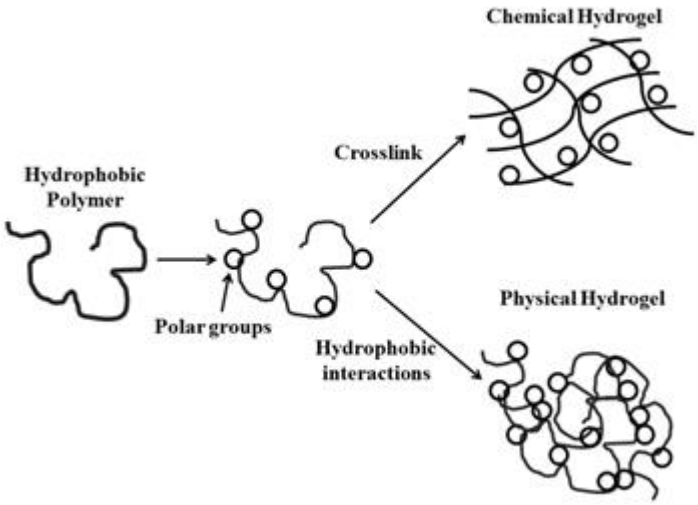


Fig 1.2 Schematic of methods for the formations of the two types of hydrogels, chemical or permanent and physical or reversible [51].

Hydrogels can be generated from a wide range of polymers, and they are characterised according to the source of these macromolecules: synthetic, natural, or a combination of both (Table 1.2) [51].

Synthetic polymers allow the control, at the molecular level, of the network structure's properties and chemical or biological responses during their design and manufacture [52, 56, 57]. Within the synthetic category of hydrogels, those most commonly employed for biomedical applications are the neutral ones. These are derivatives of PHEMA [12, 58], PVA [56, 59, 60], or PEG. PEG is probably the polymer most investigated and employed for biomaterials purposes, in view of its non-toxicity, non-immunogenicity and, also, its approval by the FDA [13, 61]. Immune alterations on PEG hydrogels, such as surface modification via covalent bonding with silicone acrylate and thiol linkages, adsorption and/or ionic attractions or hydrogen bindings, have been proposed in order to facilitate their suitabilities for cell contact [62].

Furthermore, an increased extent cell contact can be achieved by the formation of block of co-polymers such as tri-blocks of PEG with PEO, which confer degradability potentials to PEG hydrogels, or tri-blocks of PEG with PLA, or similar polymers that give rise to/ enhance the specific properties of PEG hydrogels [56].

For example, Molina et al [63] prepared a series of hydrogels from pre-synthesised PLA/PEO/PLA triblock polymers via a phase separation technique, involving the introduction of low water levels over co-polymers present in a biocompatible organic solvent system, specifically PEG-THF. The hydrogels generated in this manner exhibited hydrophilic characteristics which were greater than those of corresponding hydrogels produced via the swelling of dry tablets or films derived from these co-polymers. Indeed, they were sufficiently soft to permit their trocar injection. Moreover, both albumin and fibrinogen could be successfully entrapped in these hydrogel formulations, via their pre-mixing with the copolymer solutions prior to gel production. In terms of the time-dependent release profiles of these unique formulations, these researchers' findings were consistent with gel-protein compatibilities and incompatibilities with regard to albumin and fibrinogen, respectively [63].

As expected, hydrogels from natural sources are 'friendlier' to the user since they are non-toxic, biodegradable and biocompatible, but unfortunately lack some of the mechanical properties required [64, 65]. The most common natural sources are collagen, hyaluronic acid (HA), alginate, agarose and chitosan [53, 66].

Table 1.2 Hydrophilic polymers employed for hydrogel production [51]. See Abbreviations for definitions of terms

Polymer	
Synthetic	PEG-PLA-PEG
	PEG-PLGA-PEG
	PEG-PCL-PEG
	PLA-PEG-PLA
	PHB
	P(PF-co-EG) 6 acrylate end groups
	P(PEG/PBO terephthalate)
	PEG-bis-(PLA-acrylate)
	PEG-6CDs
	PEG-g-P(AAm-co-Vamine)
	PAAm
	P(NIPAAm-co-AAc)
	P(NIPAAm-co-EMA)
	PVAc/PVA
	PNVP
	P(MMA-co-HEMA)
	P(AN-co-allyl sulfonate)
	P(biscarboxy-phenoxy-phosphazene)
	P(GEMA-sulfate)
Natural	Hidroxyapatite
	Alginic acid
	Pectin
	Carrageenan
	Chondroitin
	Sulfate,
	Dextran sulfate
	Chitosan
	Polylysine
	Collagen (and gelatin)
	Carboxymethyl chitin
	Fibrin
	Dextran
	Agarose
	Pullulan

Table 1.2 Hydrophilic polymers employed for hydrogel production [51]. See Abbreviations for definitions of terms (continuation)

Composites	P(PEG-co-peptides)
	Alginate-g-(PEG-PPO-PEG)
	P(PLGA-co-serine)
	Collagen-acrylate
	Alginate-acrylate
	P(HPMA-g-peptide)
	P(HEMA/Matrigel)
	HA-g-NIPAAm a

The first three sources of these materials are components derivatives of the mammalian extracellular matrix (ECM), collagen being its major protein [9], and HA is a polysaccharide (consisting of linear repeating glucuronate/N-acetylglucosamine disaccharide units) which has unique viscoelastic properties and is present in almost all animal tissues. Agarose, alginate and chitosan are isolatable from marine sources and have low toxicities and a high level of biocompatibility [56].

Collagen-based hydrogels can be synthesised without chemical modifications and, in view of the presence of many cell-signal domains, they are suitable for cell growth matrices. In addition, collagen is degraded *in vivo* by enzymes, such as collagenase, which is useful for tissue engineering scaffolds or injectable systems. Nevertheless, collagen hydrogels have poor mechanical properties, but these may be improved by chemical cross-linking, cross-linking with UV light or temperature, or by the generation of admixtures with other polymeric agents [56, 66]. HA is a glycosaminoglycan associated with wound healing, and is present at high levels in joints. HA hydrogels can be produced via multiple chemical modifications, and their *in vivo* degradation is achievable by enzymes such as hyaluronidase [66–68]. Alginate hydrogels are composed of various polymer chains, the structural integrity of which is maintained by ionic bridges and divalent cations. The network cross-linking density depends on the particular monomeric units and molecular mass of the polymer. The alginate hydrogel degradation occurs by modifications of its mechanical properties [69, 70]. Chitosan is composed of linear polysaccharides obtained from the partial hydrolysis of chitin and is degraded, *in vivo*, by lysozymes. Chitosan hydrogels maintain the beneficial bioactive properties of chitin, in terms of biodegradability and bioactivity, and provides improvements in water retention capacity over that of chitin [56, 71, 72].

Composite hydrogels arise from the incorporation of natural and synthetic polymers into the same network. The 'natural' component of the hydrogel contributes to the high affinity and specificity for the cell binding activities of the matrix, and the synthetic part allows the control of the mechanical and physical properties of these systems. For example, bioactive molecules can be 'entrapped' into the synthetic network and be released by environmental stimuli, act as degradable 'trigger', or improve the biocompatibility of the system [56].

Currently, there are a number of hydrogels brands available for wound-dressing purposes already commercialized, especially in the case of skin substitutes, as reviewed by Boateng et al and Shai and Maibach [66, 73]. These skin substitutes involve scaffolds capable of releasing drugs which improve the cell growth and promote tissue regeneration. The attractive scaffolding properties of bioactive synthetic hydrogels have arisen their exact molecularly-customised biofunctions and attenuatable mechanical properties, together with the provision of microenvironments with functional properties similar to those of ECMs, which promote cell growth and tissue generation. Indeed, a range of design strategies have been examined in order to generate synthetic hydrogels with novel bioactive ECM-mimetic attributes, including cell adhesion, binding of growth factors, and proteolytic degradation [74]. Moreover, essential knowledge regarding these materials can be employed for the development of new wound dressing materials.

Hydrogels are, also, cheap and effective wound-dressing agents in view of their high level biocompatibility, permeability to oxygen, powerful water absorption and moisture retention properties (Fig 1.3). Their removal from the wound sites can be made with minimal pain or trauma [75, 76]. Moreover, they are semi-transparent, allowing visual observation of the wound state without removal of the dressing. Since these hydrogels have soothing and absorption characteristics, they are suitable for partial-thickness wounds such as superficial thermal burns, friction blisters, chemical peels, derma-abrasion, facial laser resurfacing, and ulcers [53, 77].

However, with regard to the bioactive agent delivery systems, hydrogels have a number of limitations. Indeed, the capacity of hydrogels to load hydrophobic drugs is restricted, and the release of those molecules, usually, occurs via a rapid non-linear diffusion to the surrounding environment [78]. Furthermore, the drug's biological activity may be lost via its interactions with solvents, or by phenomena imposed by the environmental conditions required for hydrogel production, such as pH and temperature [13].

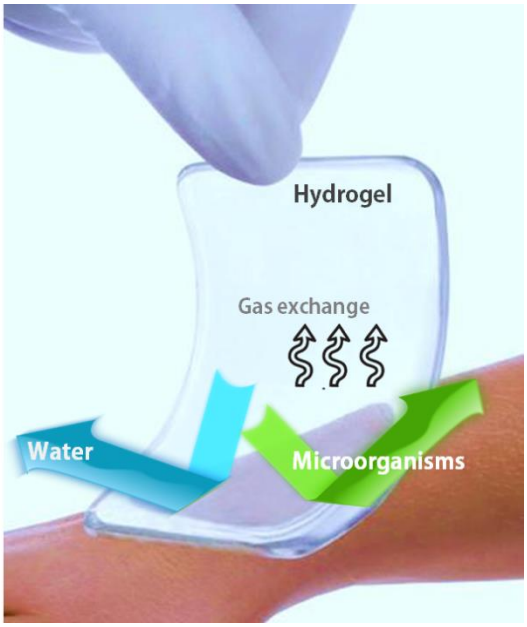


Fig 1.3 Representation of the most important features of the hydrogel as a wound dressing.

Hydrogels already commercialised included synthetic, natural and composite materials [66, 73]. However, to the best of the authors' knowledge, there are little or no publications available regarding CD-based hydrogels in which the CD species works, simultaneous, as monomers for network synthesis and as carriers for wound-dressing applications.

Supramolecular Structures of CDs for Drug Delivery Purposes

As referred above, CDs and their derivatives have been employed and studied for molecular recognition, drug delivery systems, and, also, as building blocks for nano-structured functional materials. Molecular architectures, including oligomers, polypseudorotaxanes, polyrotaxanes, nanoparticles, nanocages and hydrogels, have been constructed based on the ability of CDs to specifically link (covalently or non-covalently) to other CDs or polymers [15, 18, 79]. These nanosize structures are composed by CD aggregates with numerous functional groups, resulting in multiple cavities with the capability to interact with various 'guest' molecules. This promotes the connection between the 'guest' and the CDs by co-operative binding, which facilitates interactions between CDs and the guest's functional groups, and/or the 'guest' itself. Hence, supramolecular structures-(SM) mimic the co-operative multimode, multipoint binding often found in biological systems. Therefore, SM improve the capacities of CDs' to encapsulate drugs, a process which enhances drug bioavailability and bioactivity [80, 81].

SM-CDs can be produced by covalently linking several CD cavities on a polymer, or by grafting them on a polymer through nucleophilic displacement, condensation or acylation reactions. They may also be constructed by the contribution of several covalent interactions which give rise to well-organized structures [82].

Bioactive CD-Oligomers (O-CD) are composed by CD cavities linked by functional bridges that provide additional interactions with the 'guest' molecules, together with the co-operative binding of several CD units in order to improve the encapsulation of important bioactive 'guests' [81–85].

Polypseudorotaxane and polyrotaxane structures consist of a long-chain molecule (axle component) and several CDs (wheel components) [80, 86]. Harada et. al [82], described a method to generate both classes of these structures. The production of polypseudorotaxanes (Fig 1.4 a) involves the entrapment of CDs on polymers or polyelectrolytes, and then stabilising them through the use of hydrogen bonds between adjacent CD cavities and further non-covalent interactions between the long-chain molecule and the threaded cavities. The polyrotaxanes (Fig 1.4 b) are obtained from polypseudorotaxanes to which bulk terminals (organic or organometallic groupings) were added at the chain ends, in order to prevent the CD's detachment [81, 82].

Fig 1.4 Schematic representation of (a) αCD PEG polypseudorotaxanes and (b) αCD PEG polyrotaxanes [87].

The bioactive two- or three-dimensional supramolecular assemblies are composed by (1) an improvement of polypseudorotaxanes with added side-chains, giving the structure a two-dimensional configuration that enables interactions between SM molecules and the bioactive agent; (2) nanostructures of modified CDs with three-dimensional configurations that are constructed via covalent and non-covalent linkages with gold, and (3) hydrogels that arise from the combination of long polymers and CDs [81, 88].The first two structures have their major applications in the DNA field, and since it is outside of the scope of this thesis, only hydrogels will be discussed in the next section.

Cyclodextrin-based Hydrogels

As noted above, hydrogels possess novel properties, enabling them to be employed as drug delivery systems with the ability to improve wound healing, i.e., they have effective biocompatibilities and a sufficient water content. However, predominantly, such hydrogels require covalent cross-linking or severe environmental conditions in order to achieve a 'gel' state which limits their applicability, and, hence, the sorption of drugs is very time-consuming with these systems and impairs drug integrity [52, 79].

Therefore, the main goal of this research field is the development of a hydrogel capable of delivering a drug without an associated cross-linking agent (i.e. one that is integral and has an effective level of activity), as well as a requirement to be generated at a suitable temperature and pH value for *in vivo* use.

The further utilisation of CDs' as monomer improve the 'swelling' properties of the hydrogels and further assist in protecting the drug trapped inside the cavity (Fig 1.5) [79, 80]. Consequently, SM systems composed by polymers and cyclodextrin have been investigated for possible wound

dressing applications, since these systems have the capacity to form spontaneous tri-dimensional physical cross-linking macromolecular networks that correspond to a hydrogel [52, 89].

To date, there are little or no documented reports available concerning the actions and efficacies of CD-based hydrogels for wound dressing purposes. However, Lee et al [90] investigated the therapeutic capacity of a hydrogel composed of βCD, PEI and SF, and containing *Centella asiatica* extract and HCA in the healing of pressure sores. In view of its hydrophobic nature, HCA's release was influenced by its partitioning between the βCD cavity and the bulk water phase, and not by the hydrogel's swelling capacity. Results obtained revealed that the pressure sores treated with this hydrogel system healed within a period of 6 days, compared to a 10 day length for control (untreated) pressure sores, and the researchers concluded that the βCD/PEI/SF hydrogel containing *Centella asiatica* extract and HCA diminished the healing time required for these sores [90].

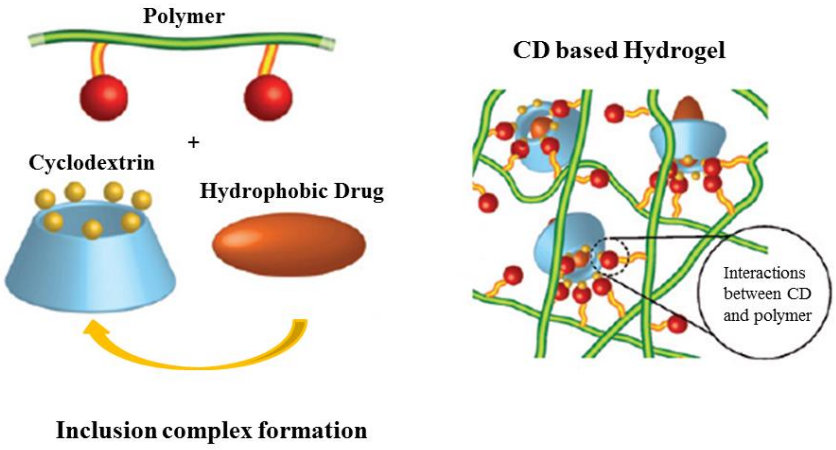


Fig 1.5 Schematic representation of the CD-based hydrogel, where CD may have two utilities: (1) monomers and (2) a drug carrier. This system improves the capacity of the hydrogel to carrier hydrophobic drugs and avoids the drug aggregation (adapted from Peng et al [91]).

CD-based hydrogels result from the 'threading' of CDs onto parts of long polymers or copolymers with high molecular masses. The CD-drug complex may be added to the polymer (1) after or during gel cross-linking, (2) via grafting, or (3) by direct cross-linking with digylcidyl-ethers. The first method is easier to perform but may induce an early liberation of the complex from the hydrogel, alleviating the extent of control over the release kinetics. On the other hand, the other two strategies permit a better level of modulation of drug release from the system [52].

Chemical supramolecular hydrogels are typically formed from polypseudorotaxanes or polyrotaxanes with CDs covalently-linked by cross-linking agents, or by modifications of the polymeric backbone. For example, biodegradable polymers with hydrolysable traps have been developed for drug or gene delivery applications [92].

The first published work with CD-containing polymeric hydrogels described a procedure to build a network from the chemical cross-linking of α, β or γCDs with epichlorohydrin (EPH), as a biofunctional cross-linking agent [93]. The first study proved that this network was able to form a complex with a variety of poorly water-soluble drugs. To enhance the swelling capability, increase the drug loading capacity and reduce the toxicity of EPH, the cross-linking reaction was performed in the presence of cationic or anionic compounds. For example, Li et al [94] described a positively-charged network by cross-linking βCD with EPH in the presence of choline chloride, and this resulted in an effective encapsulation, and release of the anti-inflammatory drug naproxen and had a lower toxicity when compared with networks without CDs. Negatively-charged EPH cross-linking βCD networks containing carboxymethyl groups were also reported. In this case, the hydrogels could be loaded with cationic drugs, such as those with microbicidal properties, a phenomenon rendering them useful for wound dressing and chewing gum formulations of value against mucosal infections. Other cross-linking agents were also described for CD-containing polymeric hydrogels, such as diepoxides, alkyleneglycoldi (epoxypropyl) ethers, the di-isocyanate hexamethylene diisocyanate, and anhydrides. The use of CD derivatives, such as MβCD, SBβCD or HPβCD, has been a helpful strategy, since they are capable of improving the capacity of these hydrogel materials to absorb water [18].

In order to tailor the mechanical properties of covalently-linked networks, this cross-linking has been employed to incorporate water-soluble polymers, such as PVA or hydroxypropyl methycellulose (HPMC). Nozaki et al [95] described a procedure to couple pNIPAAm-, (with terminal carboxyl acid groups) to amine-functionalised EPH pre-cross-linked by βCD. However, the material obtained exhibited a temperature-dependent behaviour.

Although polymerisation of CDs with low-molecular-mass cross-linking agents demonstrated appropriate properties, the coupling of pre-existing networks to modified or unmodified CDs achieves an improvement in these properties. For example, CDs can be used as cross-linking agents as described by Bibby et al [96]. Their work reported an esterification of CD's OH with PAA carboxylic acid groups, and generation of an anhydride between PAA chains in this manner

led to the formation of a network inducible with temperature. Paradossi et al [97] suggested the production of a heterogeneous biocatalyst gel system loaded with copper(II) ions, by cross-linking chitosan with an oxidised (aldehyde-containing) CD via reductive amination. A recent work used a highly-selective copper(I)-catalysed, 1,3-dipolar cycloaddition (click chemistry) between an alkyne-modified CD and an azide-functionalised poly(NIPAAm-co-HEMA) during hydrogel generation. The improvements offered by this process were an effective control of the gelation rate and the reaction conditions [98, 99].

Copolymerisation and chemical- or radiation-mediated inductions are also widely used for the production of CD/polymer networks. The most common polymers used are vinyl- or (meth)acryloyl-modified CD monomers with further vinyl monomers (acrylic acid, 2- HEMA, and NIPPAm). γ-CD-PAA systems with pH- dependent swelling properties [100], and βCD-maleic anhydride hydrogels with both temperature- and pH-dependent behaviour [101] have been described for drug-release materials.

Moreover, vinyl- substituted polymeric macromers, such as MAH and PLA, the MAH-substituted block copolymer of Pluronic F68, and PCL have been employed for the development of hydrogels with controlled characteristics via radical polymerisation with CD derivatives [102].

Covalently-linked polymer/CD adducts can also be developed by cross-linking CD to reactive polymer end-groups, for example end-modified PEG, and these agents have swelling properties which depend on the network composition (i.e., the ratio of polymer:CD). Desirable PEG characteristics (biocompatibility, non-immunogenicity, and an effective loading capacity for hydrophobic drugs) render these hydrogels valuable candidates for biomaterials [103, 104].

In order to avoid the adverse effects of using cross-linking agents for hydrogel formation, selected systems have been developed in which gelation is induced via complexation between CDs and polymer chains [18, 79].

Physical supramolecular hydrogels arise from non-covalent interactions, such as (1) electrostatic interactions, (2) hydrophobic interactions between amphiphilic polymers, (3) hydrogen-bonding or (4) stereo complex formation between polymers with opposite chiralities, which confer reversibility behaviour to the network systems. This cross-linking can be performed in aqueous environments, whilst the drug is loaded, a process which improves the properties of the network [18, 105].

In this case, network generation is a consequence of the CD inclusion of 'guest' molecules, e.g. linear polymers. The polymers predominantly used are PEG, PPO and PCL [105]. PEG is probably the most widely employed for physical CD-based hydrogels in view of its biocompatibility, biodegradability and hydrophilicity, properties which render the network suitable for biomedical applications [79].

Hydrogels based on α-CD-PEG and its copolymers have been designed, and modifications to these materials have been made to improve their physicochemical properties, and, also, their biocompatibilities. The triblock of PEG and PPO (PEG-PPO-PEG) has weak physical interactions between chains in aqueous environments, an observation ascribable to the hydrophilic behaviour of PEG, which renders this copolymer unsuitable for long-term drug release applications. However, this can be overcome by using αCD as a 'trap' to the PEG segments, a process which changes the hydrophobicity of the copolymer, and hence reduces the effective polymer concentration required for gelatinisation, and also confers thermo-reversibility behaviour to the copolymer [79]. Moreover, the PEG-PPO-PEG copolymer is not biodegradable, and therefore the use of PHB, a natural, optically-active and biodegradable polyester with high crystallinity and hydrophobic properties (instead of PEG only on its triblocks), gives rise to a polypseudorotaxane with a high self-assembly tendency in aqueous environments. The stability of the copolymer PEG-PHB-PEG is attributable to complementation of the complexation between the αCD and PEG, with hydrophobic interactions between the mid-placed PHB blocks. For these reasons, PEG-PHB-PEG is a thyrotrophic and reversible supramolecular hydrogel, and can be employed for the long-term release of macromolecular drugs without a requirement for post-application removal [79, 92].

Additionally, αCD-PEG-PCL, (where PCL is an amphiphilic biodegradable copolymer) presented a similar behaviour to αCD- PEG-PHB-PEG. This is specifically the case for competition between complexation of PEG by CD versus hydrophobic PCL, as well as for the reversibility behaviour of the hydrogel and its thyrotrophic properties. This shows that it is possible to develop supramolecular hydrogels by IC formation with other amphiphilic biopolymers for the purpose of drug delivery with a minimal level of adversity to the user [79, 92].

The reversible properties of these physical hydrogels result from the entropically-unfavourable inclusion of the polymer and the CD via complexation linkages. Therefore, changes in the environment, such as temperature and pH values, can induce dissociation of the polymer from the CDs [92].

Plant-derived bioactive agents

Plant extracts have been used in traditional medicine, and during the past few years many studies have proved their beneficial effects on human health. The plant extract's bioactivities are commonly related with compounds like fibre, vitamins, phytosterols, sulphur-containing compounds, carotenoids, organic acid anions, together with polyphenolics [106]. This kind of molecules is plants secondary metabolites and includes a wide range of compounds, many of which are phenolics or their oxygen-substituted derivatives (Fig 1.6). Polyphenolics confer protection to the plant, and are responsible for plant odours (essential oils), plant pigmentation (quinines and tannins) or their flavours (e.g., terpenoid capsaicin from chilli peppers) [23].

Polyphenolics are plant metabolites present in human and animal diets, with a wide range of biological activities on human body, such as anti-oxidant, anti-inflammatory, antibacterial and antiviral [25, 26, 106–109]. They share a common chemical structure: all have at least one aromatic ring, with one or more OH attached. The diversity of phenolic compounds present in nature result from variations in the basic chemical skeleton, such as degree of oxidation, hydroxylation, methylation, glycosylation, and conjugation with further molecules, particularly lipids, proteins, other phenolics, and biomolecular metabolites [25, 110].

Therefore, this group of natural bioactive compounds includes a range of molecules from simple, single aromatic-ring, low-molecular-mass compounds, to large and complex tannins and polyphenolic derivatives. However, they all share two fundamental biological activities (1) radical scavenging action and (2) anti-oxidant properties by interaction with proteins and ions [25, 111–113]. The anti-oxidant activity of lipophilic phenolics and polyphenolics can be attributed to their action as chain-terminator for the self-perpetuating autocatalytic lipid peroxidation process, as indeed does α-tocopherol (vitamin E).

Polyphenolics are grouped by the number and arrangement of their carbon atoms (Fig 1.6) [110]. Polyphenolics will be sub-divided as flavonoids and non-flavonoids.

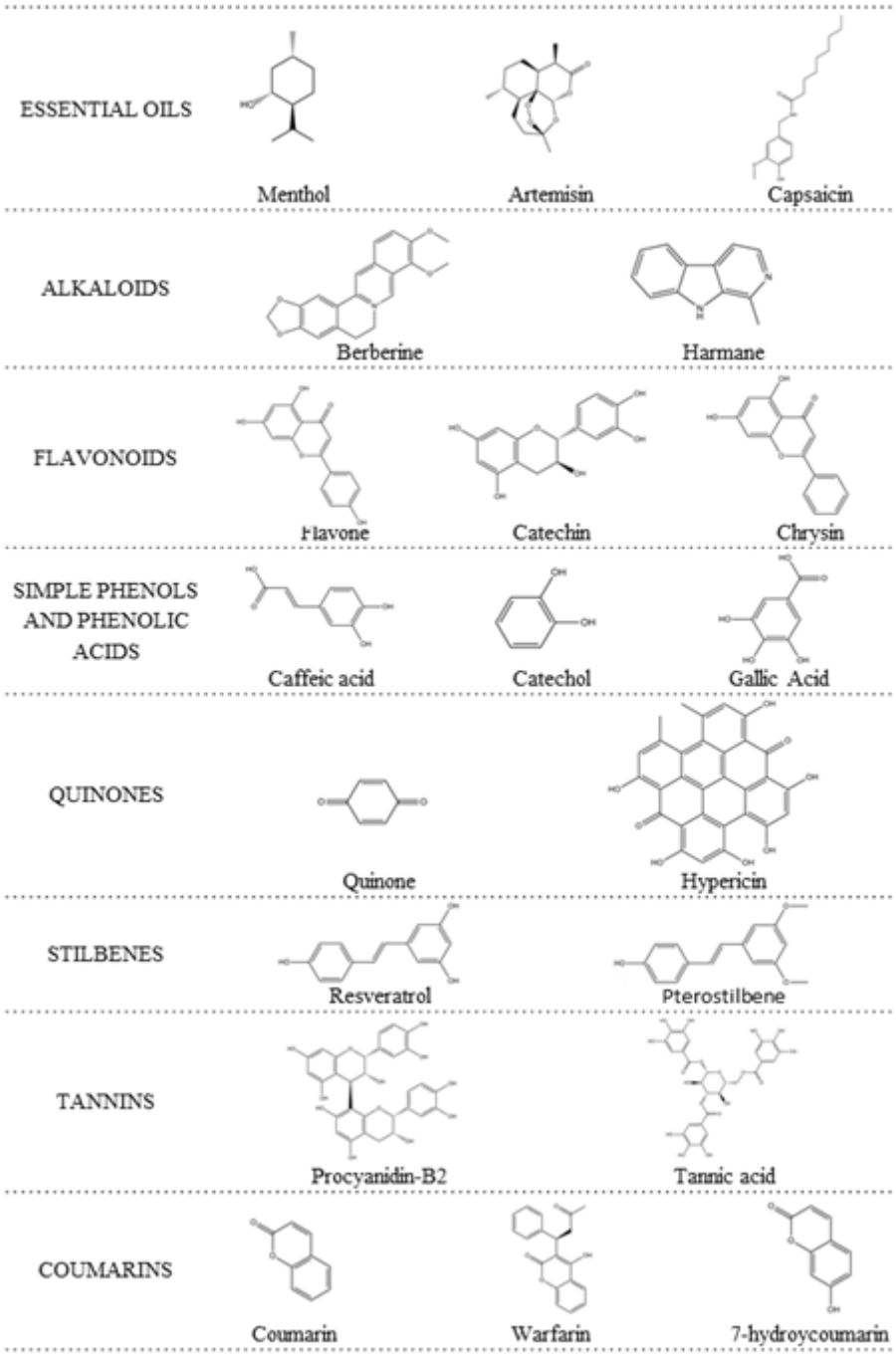


Fig 1.6 Chemical structures of common plant-derivable bioactive agents (Chem3D Pro 12.0 software).

Flavonoids

Flavonoids are low-molecular-mass compounds with a flavan nucleus: two aromatic rings connected by 3-carbon bridge (C_6-C_3-C_6) [106, 112]. In plants, they are utilised in response to microbial infection. However, in animals and humans, flavonoids protect cells against damage caused by reactive oxygen species (ROS), and, also, defend skin from damage induced by short

wavelengths [33]. Moreover, they have the capacity to inhibit the growth of a wide range of bacteria via disruption of bacterial cell walls, following by their complexation with the extracellular soluble protein components [23]. Flavonoids, also, exert anti-viral actions due to their favourable oxidation potentials [114, 115].

The heterogeneity of the flavonoid group arises from the numerous substitutional modifications possible on the basic carbon-based skeleton. The presence of OH and sugars are very common, and increase their water solubility. However, methyl groups and *iso*-pentyl units increase their lipophilicity [110]. The bioactivities and properties of the flavonoids are critical, and are affected by small changes in the chemical structures [23, 116]. Therefore, flavonoids can be divided into sub-classes according to the precise nature of their chemical substituents, summarized on Table 1.3.

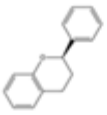
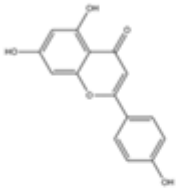
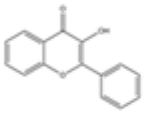
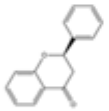
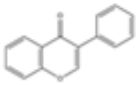
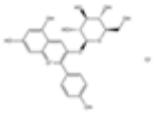
Non-Flavonoids

Non-flavonoids include all the other polyphenolics (Fig. 1.6), from the simplest phenolics and phenolic acids to the phenolic complex tannin. This non-flavonoids group incorporates (1) phenolic acids, (2) quinines, (3) stilbenes, (4) tannins, and (5) coumarins.

Although phenolics and phenolic acids are single-substituted phenolic rings, they are capable of numerous biological effects. In fact, some authors have related the number of OH on the aromatic ring with their antimicrobial activity: the higher is number of OH the higher is the level of toxicity exerted to micro-organisms [110]. Phenolic acids include derivatives of benzoic acids, i.e. (C_1-C_6)- hydroxybenzoic acids, and derivatives of cinnamic acid, i.e. (C_3-C_6)- hydroxycinnamic acids [117, 118]. Gallic, *p*-hydroxybenzoic, and ellagic acids are representatives of the first group, and frequently occur in the form of glucosides. These compounds are water-soluble and sensitive to temperature, elevated pH values, oxidation and light [25, 111, 118]. Caffeic, ferulic and *p*-coumaric acids belong to the class of hydroxycinnamic acids, and are rarely found in the free form; indeed, they are usually present in nature as simple esters with hydroxy carboxylic acids or glucoses. The hydroxycinnamic acids are, also, sensitive to oxidation and high pH values, and are poorly-soluble in water [25, 110, 111, 119]. Phenolic acids have been described in the literature as molecules with effective anti-microbial activity, particularly as fungicides. Their antimicrobial activity critically depends on phenolic chemical structure, especially on the number and position

of the substitution in the benzene ring, and, also, on the saturated chain length. The microbicidal capacity was found to be increased with augmentation of the alkyl chain length [118, 120, 121].

Table 1.3 Classification, structure and possible substitutions of the major classes of plant-derived flavonoids (Chem3D Pro 12.0 software)

Class	General Structure	Flavonoid	Substitution Pattern
Flavan-3-ols		(+)-catechin	3,5,7,3',4'-OH
		(-)-epicatechin	3,5,7,3',4'-OH
		Epigallocatechin gallate	3,5,7,3',4'-OH, 3-gallate
Flavone		Chrysin	5,7-OH
		Apigenin	5,7,4'-OH
		Rutin	5,7,3',4'-OH, 3-rutinose
		Lutiolin	5,7,3',4'-OH
		Lutiolin glucosides	5,7,3'-OH, 4'-glucose
			5,4'-OH, 4',7-glucose
Flavonol		Kaempferol	3,5,7,4'-OH
		Quercetin	3,5,7,3',4'-OH
		Myricetin	3,5,7,3',4',5'-OH
		Tamarixetin	3,5,7,3'-OH, 4'-OMe
Flavanone		Naringin	5,4'-OH-rhamnoglucose
		Naringenin	5, 7, 4'-OH
		Taxifolin	3,5,7,3',4'-OH
		Eriodictyol	5,7,3',4'-OH
		Hesperidin	5,7,3'-OH,4'-OMe, 7-rutinose
Isoflavone		Genistin	5,4'-OH, 7-glucose
		Genistein	5,7,4'-OH
		Daidzin	4'-OH, 7-glucose
		Daidzein	7,4'-OH
Anthocyanin		Apigenidin	5,7,4'-OH
		Cyanidin	3,5,7,4'-OH, 3,5-OMe
		Malvidin	

Quinones are highly-reactive, oxidised polyphenolic agents containing an aromatic ring (phenol group-oxidised) with two ketone substitutions. In plants, they are responsible for the brown colouration of the injured fruits, and act as intermediates in the melanin synthesis pathway in humans. Furthermore, quinones exert powerful antimicrobial activities, by linking irreversibly to proteins and enzymes of the surface wall and membrane of microorganism, and thereby inactivating them. However, this mechanism of action may also be responsible for their toxicological actions in humans [23].

Resveratrol serves as a typical representative of the stilbenes group. The members of this group are characterized by a C_6-C_2-C_6 structure, and are produced by plants during episodes of stress, such as those arising from disease or injury [110, 111, 122]. Stilbenes, specially resveratrol, have been established anti-ageing and anti-oxidant [116].

Tannins are a group of relatively high-molecular-mass biomolecules capable of tanning leather, or precipitation of gelatine from solution. Tannins based on a gallic acid precursor or 'nucleus' can be hydrolysable as multiple esters with *D*-glucose, or based on condensed derivatives from flavonoid monomers, also known as proanthocyanidins. Tannins have the capacity to stimulate phagocytic cells, and also act as host-mediated tumour suppression or microbicidal agents. The latter activity results from the capacity of these agents to reversibly bind to proteins via hydrogen bonding and/or hydrophobic interactions (van der Waal's forces), or irreversibly via covalent bonding processes which inactivate the enzymes and adhesins present on the microbial cell wall [23, 111].

Coumarins include phenolic agents with fused benzene or α-pyrone rings; indeed, the basic structure can provide a wide range of substitutional modifications which modulate their biological activities. The major bioactivities assigned to this group of compounds are antithrombotic, anti-inflammatory, anti-allergic, hepatic-protective, antiviral, anticarcinogenic, and vasodilator agents [123, 124]. Warfarin, [2-hydroxy-3-(3-oxo-1-phenylbutyl)chromen-4-1], serves as a good example of a coumarin-based drug available with good anticoagulant and antiviral properties [23].

Cyclodextrins and Polyphenolics

The number of currently-available pharmaceutical products based on polyphenolic agents is enormous, and its relevance to the global economy is consistently growing. A large number of plant extracts and their constituents, already employed in the food industry, have been adapted to serve as major active ingredients in both cosmetic and health products. However, the effectiveness of these active compounds deepens the preservation of their stability, bioactivity and bioavailability [26]. Indeed, limited water solubility, differences in the amounts of extract required for bioactive effects, and the rapid oxidation of at least some of these agents, represents some of the problems detected during the developmental stages of drugs based on polyphenolics [25, 26, 36]. Therefore, new approaches have been developed in order to overcome these drawbacks. Indeed, their encapsulation with CD species is one of them.

A wide range of reports have been published regarding the encapsulation of natural polyphenolic agents by CDs, for food and drug delivery proposes. A brief resume of the available published work regarding the IC between the most common polyphenolics and CD with pharmaceutical applications will be made. The works with IC polyphenolics-CDs are summarized on Table 1.4 and some of them are analysed with more detail.

Table 1.4 Published works regarding the inclusion of polyphenolics by CDs and derivatives

	Polyphenolic	CD	Improved characteristics	References
Essential Oils	Eugenol	βCD		[125]
	Lavender	β-D	Solubility	[126]
	Mint	βCD		[126]
Alkaloids	Curcumin	αCD	Solubility	[127]
		HPαCD	Anti-carcinogenic	[128]
		βCD	Transdermal permeation	[129]
		HPβCD	Anti-inflammatory	[130]
		RMβCD	Bioavailability	[131]
		SBEβCD	Photodegradation	[132]
		HTAβCD		[133]
		HPγCD		[134]

Table 1.4 Published works regarding the inclusion of polyphenolics by CDs and derivatives (continuation)

Alkaloids	Curcumin	γCD		[135] [132]
Phenolic acid	Caffeic acid	βCD HPβCD	Solubility	[125] [136]
	Catechol	βCD		[125] [137]
	Chlorogenic acid	βCD	Solubility, Anti-oxidant, Antimicrobial	[49]
	Coumaric acid	βCD	Solubility, Anti-oxidant	[138]
	Ferulic acid	αCD βCD γCD	Solubility, Photostability, Transdermal permeation	[125] [139] [140],[141]
	Nerolidylcatechol	HPβCD	Solubility	[142]
	Rosmarinic acid	βCD HPβCD HEβCD MβCD	Solubility Anti-oxidant	[143]
	Vanillin	βCD		[125]
Stilbenes	Resveratrol	αCD βCD HPβCD MβCD DMβCD	Thermal stability Solubility Anti-oxidant Anticarcinogenic UV degradation	[144] [145] [146] [147] [148]
Flavon-3-ols	Epigallocatechingallate	βCD HPβCD DMβCD	Solubility Anti-oxidant	[149]
Flavone	Apigenin	DMβCD HPβCD	Solubility	[150]
	Baicalein	αCD βCD HPβCD DMβCD γCD	Solubility Thermal Stability	[151]

Table 1.4 Published works regarding the inclusion of polyphenolics by CDs and derivatives (continuation)

Flavone	Chrysin	βCD DMβCD HPβCD	Solubility Anti-oxidant	[152] [150]
	Luteolin	βCD MβCD DMβCD HPβCD HEβCD G_2βCD	Solubility	[153] [150]
Flavonol	Rutin	HPαCD βCD HPβCD γCD HPγCD	Solubility Stability Anti-oxidant Bioavailability	[154] [155] [156] [157] [158]
	Catechin	βCD	Solubility, Anti-oxidant, Transdermal permeation	[159], [160], [161]
	Galangin	HPβCD DMβCD	Solubility	[162]
	Isoquercetin	βCD HPβCD DMβCD		[163]
	Kaempferol	βCD G_2βCD HPβCD DMβCD	Solubility Stability Thermal Stability Anti-oxidant	[164] [165] [162] [166]
Flavonol	Myricetin	HPβCD DMβCD	Solubility Anti-oxidant	[162] [166]
	Quercetin	βCD HPβCD MβCD DMβCD	Solubility Anti-oxidant Photostability	[159] [158] [162] [33] [166]

Table 1.4 Published works regarding the inclusion of polyphenolics by CDs and derivatives (continuation)

Flavanone	Alpinetin	HPβCD	Solubility, Stability	[167]
	Astilbin	αCD βCD γCD	Solubility	[168]
	Naringenin	βCD HPβCD DMβCD TMβCD	Solubility Bioavailability	[169], [170], [47]
	Naringin	βCD	Solubility	[170]
	Hesperetin	βCD	Solubility	[170]
	Hesperidin	βCD	Solubility	[170]
Isoflavane	Daidzein	βCD HPβCD RM-βCD γCD	Solubility Bioavailability	[171], [172]
	Genistein	βCD HPβCD RMβCD γCD	Solubility Bioavailability	[171], [172]
	Glycitein	βCD HPβCD	Solubility	[171]
	Puerarin	G_2βCD	Solubility	[173]

Cyclodextrins and Flavonoids

The majority of the publications in this area of expertise concern the encapsulation of flavonoids with βCD and its derivatives in order to improve the flavonoids water solubility and stability.

The catechin, epicatechin and epigallocatechin-gallate are molecules representative of the flavon-3-ols subgroup (Table 1.3). These compounds present antidiabetic and antiobesity properties, besides the anti-oxidant action [174]. Catechin isolated from grape seed was successfully complexed with βCD with a 1:1 stoichiometry [160]. Moreover, the solubility profile of epigallocatechin after encapsulation with βCD, HPβCD and DMβCD was described by Folch-Cano et al [149]. They observed that the temperature had a different influence on the K, dependent on the CD used. In the case of the native CD, the K rise with temperature increase, the opposite was

observed for the CD derivatives used in this study. Hence, the DMβCD was the most suitable CD for the complexation of epigallocatechin. The authors also described that regardless of all the IC had similar geometries, the flavonoid anti-oxidant rings position inside the CD cavity was different [149].

Rutin is a flavone used in capillary preservation drug with poor solubility. The encapsulation of this polyphenol with αCD, βCD, HPβCD and DMβCD was described by different authors in order to improve rutin solubility, and, consequently, pharmacological activity [156, 158, 175, 176]. For the CDs used, the IC formed was 1:1, this means that each molecule of cyclodextrin was capable of interact with one molecule of rutin. Haiyun et al [175], Shuang et al [156] and Sri et al [158] achieved similar K for the IC of rutin and βCD (between 260 to 265 M^{-1}).

Regarding HPβCD, the IC formed was more stable since the constants described were higher than the ones for the βCD/rutin. The constant value achieved by Sri et al was a lower than Shuang et al, meaning that higher temperatures (28°C in the first case) are less favourable for this IC formation. However, the DMβCD was the CD with higher K (3217.62 M^{-1}) [176] and the αCD with lower capacity to complex with rutin, probably related to the CD' cavity size [156]. The formation of IC improved rutin solubility and, consequently, it anti-oxidant activity and bioavailability. For instances, the complexation of rutin with βCD improved it anti-oxidant protection of cells against oxidative stress [154]. The enhance of the oral availability by the capsulation of rutin by HPβCD was proved by Miyake et al [155] after administration of the ICs to beagle dogs. The stability of rutin can be improved by complexation with HPβCD or HPγCD, the hydroxypropyl groups enhanced the interactions stability of the CD with rutin and the analyse of NMR showed that the rutin A ring was inside the cavity of HPβCD. The IC protect rutin from thermal and UV degradation and, also, increased this phenolic anti-oxidant capacity [157].

Chrysin is also a flavone and its pharmaceutical applications are related with its anti-oxidant, anti-inflammatory and antihypertension capacity. However, as most part of this kind of compounds, chrysin has low solubility in water. Therefore, the use of CD as carrier agent improved it activity and administration [152]. Chrysin IC with βCD were investigated by Chakraborty et al [152]. The complex had the stoichiometry of 1:1 and with a K of 1005 M^{-1}, anti-oxidant activity of the chrysin was also improved [152]. Kim et al [150] encapsulate this molecule with HPβCD, DMβCD, besides the native CD. They report the same stoichiometry (1:1) for all the CD use and the HPβCD had the higher K (1855 M^{-1}) [150]. In both works, authors described that the interaction

between the chrysin and the CDs occurs by the A-ring of the flavon, molecular interactions already mention for rutin [150, 152].

As the most part of flavonoids, quercetin is flavonol with antibacterial, anti-oxidant and antitumor properties, but its use on the pharmaceutical field is limited due to their sparingly solubility in water [177]. The βCD was used to improve solubility and photo stability of quercetin by several authors [158, 177, 178]. Jullian et al [177] and Calabro et al [178] reported a 1:1 stoichiometry complex, but Sri et al [158] described a 1:2 complex. As it was mention above, in the same equilibrium, it is possible to find different interactions between CD and the guest molecule (1:1 or 2:1). Moreover, the K values, assessed by these authors, rise with the increase of the temperature. Jullian et a. [177] used 30°C to prepare the βCD/quercetin and had the higher stability constant (602 M^{-1}). In the same work, the IC formation with SBEβCD and HPβCD was, also, established. The IC for both βCD derivatives with quercetin was 1:1 and the solubility of quercetin was enhanced, with K of 4032 and 1419 M^{-1}, respectively, without diminishing its anti-oxidant property. Moreover, the CD derivatives were more efficient on the solubilisation of quercetin than the natural CD and showed better anti-oxidant activity [177].

The HPβCD was, also, used by Sri et al [158] and by Mercader-Ros et al [166] to enhance the solubility and anti-oxidant properties of the quercetin. In both situations, the K value found was lower (321 and 900 M^{-1}) than the mentioned by Jullian et al, as well as the temperature used (28 and 25°C) [158, 166]. This means that the temperature is a crucial parameter on HPβCD/quercetin IC formation. The anti-oxidant activity of the quercetin was improved by its encapsulation by βCD derivatives (HPβCD, SBEβCD, DMβCD and MβCD) [33, 166, 176, 177]. The enhacment of quercetin biological activity may be a outcome of the protection from the rapid oxidation, by free radicals, conffered by the CDs [166]. Carlotti et al [33], also, reduced the photodegradation ratio of the quercetin by its complexation with MβCD. They claimed that because quercetin was in an apolar environement inside the CD cavity the photolytic reaction was reduced, and the amount of light capable of reach this flavonol was lower, since it had to cross the CD molecule [33].

Kaempferol is, also, a flavonol with great interest in the pharmaceutical field due to its potent anti-oxidant activity [179]. Its behaviour in aqueous environment had been improved by the utilization of CDs as encapsulating agents. As the other works described above, the βCD derivatives, such as HPβCD, DMβCD and G_2βCD, were more suitable for the IC formation with

kaempferol than the βCD [162, 164–166]. Additionally, the temperature determines the K values, but in this situation IC formation was improved by lower temperatures [165]. The beneficial effect of the IC on the anti-oxidant activity of kaempferol was determined by Kim et al [162] and Mercader-Ros and et al [166].

The naringenin is a flavanone with a similar structure of the rutin, with good anti-oxidant capacity and capable of reduce the cholesterol plasma level [180]. The formation of IC between naringenin and βCD and its derivatives (HPβCD, DMβCD, MβCD and TMβCD) was analysed by several authors [47, 169, 170]. For the CDs mentioned above the stoichiometry of the IC was 1:1, the HPβCD was the CD with higher stability constant [47]. Yang et al [169] demonstrated that the water solubility and thermal stability of this flavonoid was improved when encapsulated by β-CD, DMβCD or TMβCD. In fact, the ICs remained stable when exposed to temperatures near 225°C. Based on their analysis of the NMR, they assumed that the C-ring of naringenin was interacting with the CDs' cavity [169]. The biological effect of the IC HPβCD/naringenin was also described. Shulman et al [47] proved that the solubility of the flavonoid was increased 400 times when complexated with the HPβCD. Moreover the naringenin transport across the model of the gut epithelium (Caco-2 cells) was, also, enhanced as well as it plasma concentration. Therefore, the IC HPβCD/naringenin can be used as oral delivery drug for the treatment diabetes and dyslipidaemia [47].

Ficarra et al [170] and Tommasin et al [181] investigated the effect of the βCD and HPβCD IC on the solubility of the flavanones hesperetin and hesperidin. In the first work, hesperetin and hesperidin were complexed with βCD and improvement on the solubility and chemical stability were demonstrated [170]. Tommasin et al [181] demonstrated that the ICs formed was 1:1, and the better complextion was observed by HPβCD and hesperetin, based on the higher K values (21000 M^{-1}) when compared to the ones assessed for hesperidin (K 90 M^{-1}). The K values discrepancy can be justify by the size of the flavanones molecules, the hesperetin is smaller and less polar which make it more appropriated to interact with the hydrophobic cavity of the CD derivative. Nerveless, the solubility of both flavanones was improved and, consequently, their anti-oxidant activity. Therefore, the hesperidin and hesperetin application as anti-oxidant and anticarcinogenic can be upgraded by the use of HPβCD as drug delivery agent [181].

Yang et al [119] used the three native CD and HPβCD for the IC formation with taxifolin, a flavanonol able to dilates bloods vessels, enhance microcirculation and cerebral blood flow and

prevent platelet aggregation. In this investigation, they proved that the ICs formed enhanced the taxifolin water solubility and thermal stability, highlighting their use on healthcare products. Moreover, βCD showed better capacity to interact with this flavanonol [119].

The genistein is an isoflavone used on treatment and prevention of estrogenic related cancers or postmenopausal symptoms, due to its great affinity to estrogenic receptors. This polyphenolic has, also, anti-inflammatory effects and platelet aggregation inhibition. However, genistein limited solubility reduce its applicability in the pharmaceutical industry [172]. The complexation of genistein with CD can improve its solubility and enhance its biological effects. Daruházi et al [172] tested the IC formation between this isoflavonoid and βCD, γCD, HPβCD and RMβCD. The genistein was capable of interact with the 4 CD, but the CD derivatives induced a higher influence on the solubility of the compound. The genistein capacity to cross biological membranes was, also, improved by the encapsulation with the CDs [172]. Yatsu et al [171] assessed the βCD and HPβCD encapsulation capacity of a mixture of daidzein, genistein and glycitei. All the isoflavones showed higher affinity to the HPβCD, and the interaction between the CD and the bioactive molecules occur by introduction of the B-ring into the CD cavity, besides external interactions [171].

Based on the works described above, the derivatives of βCD are more appropriate for the improvement of the flavonoids solubilisation and stabilization, being the HPβCD the most used. These group of flavonoids can interact with CDs by (1) directing the B-ring toward the secondary rim of the CD or (2) heading the A-ring toward the secondary rim of the CD [162]. Moreover, flavonoids photo and thermal stability upgrade by encapsulation with CDs, as well as their anti-oxidant activity, since the CDs prevent the oxidation by free radical of the flavonoids.

Cyclodextrins and non-flavonoids

As referred above, the non-flavonoids had great importance on the pharmaceutical and cosmetic industry due to their biological properties. Though, their application is limited because these polyphenolics are very susceptible to degradation by environmental factors such as light, temperature and pH [110, 182]. In order to overcome this, some works have been published regarding the inclusion of non-flavonoids into CD.

The ferulic acid is, commonly, used for the preventing UV light induced skin tumour, but it has low stability under thermal and physical stress. The IC formation between ferulic acid and αCD was investigated by Anselmi et al [140]. Thus, the authors proposed the inclusion of this phenolic acid into the αCD, with a K value of 1162 M^{-1} and equimolecular complexation. Based on their results, the α and β unsaturated part of the ferulic acid and part of its aromatic skeleton were inside the hydrophobic cavity of the CD. The IC increased the ferulic acid resistance to the degradation by UVB and also, decreases it rate release [140]. The same phenolic acid was used by Casolaro et al [141] for the complexation with αCD. The stoichiometry of the IC was the same and they assumed that the ferulic acid was neutralized inside the CD cavity [141]. The cutaneous permeation and distribution through skin of the IC ferulic acid and αCD was assessed by Monti et al [139]. They concluded that the IC prevented the formation of the less active *cis*-isomer of ferulic acid and its degradation by UV light. The αCD/ferulic acid IC presented lower penetration on the skin which enlarge the skin protection against UV damages, since the ferulic acid remains at the skin surface [139]. The encapsulation of ferulic acid with HPβCD was also studied [183]. The IC obtained had lower stability (K 166.3 M^{-1}), and the same stoichiometry of the others IC described above. Nerveless, the solubility and protection against decomposition caused by irradiation with UV light, was enhanced by the complexation of the ferulic acid with this CD [183].

Although, caffeic acid has been described as antibacterial and anti-oxidant phenolic acid, its biological activity may be jeopardized by its sensibility to oxidation and lower solubility [136]. Thus, some authors had described its encapsulation with CD to overcome these issues. Górnas et al [137] and Divakar and Maheswaran [125] complexed this phenolic acid with βCD. In both cases, the experimental results suggest a 1:1 IC with K of 270 and 516 M^{-1}, respectively. The molecular interaction was described as follows, the OH of the phenolic acid were trapped inside the βCD cavity and the carboxyl moiety was projected outwards the CD [125, 137]. Górnas et al [137] studied the influence of the pH on the IC formation, and concluded that the K decreased with the presence of caffeic acid charged species. The caffeic acid was, also, encapsulate by HPβCD to increase solubility [136]. The authors conclude that the IC formation was better in acid conditions and the IC ratio was 1:1, also, the caffeic acid solubility increase. The lipophilic aromatic ring and ethylene portion of the caffeic acid was entrapped inside the CD cavity and the polar groups were outside the HPβCD cavity (Fig 1.7) [136].

Rosmarinic acic, a hydroxycinnamic acid with high anti-oxidant properties and poor solubility, was encapsulated with αCD, βCD , HPβCD , HEβCD and MβCD [143] in order to improve both properties. By the observation on the UV-Vis spectrum of the rosmarinic acid and the ICs, the authors assessed the stoichiometry of all ICs (1:1) and the K. They reported that ability forming stables IC was as follows MβCD> HEβCD> HPβCD> βCD> αCD. Additionally, the anti-oxidant activity of the ICs was higher than the rosmarinic acid alone [143].

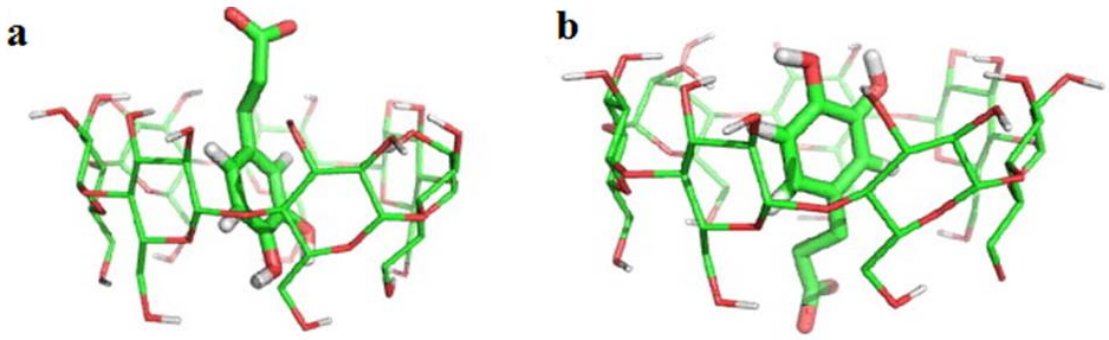


Fig 1.7 Optimised structure of the HPβCD/caffeic acid complex. Caffeic acid is inserted into the apolar cavity of HPβCD from its (a) "top" side; (b) "bottom" side.

Resveratrol *trans*-3,4,5'-trihydroxystilbene is a polyphenolic with a high level of therapeutic potential as anticarcinogenic and anti-oxidant [122]. This stilbene displays a hydrophobic behaviour, and is, also, extremely affected by exposure to oxygen, light, and oxidative enzymes, reducing its bioactivity. The use of CD to protect resveratrol and to increase it solubility, stability and bioactivity was applied in several studies [122, 144, 146–148, 184]. The effect of 3 native CDs and MβCD on the thermal stability of the resveratrol was reported by Li et al [144]. Based on the thermal analyses made, they assumed that the IC formation was favoured by the temperature rise, since all the reactions had negative enthalpy energy. The encapsulation of γCD and resveratrol was the most stable owing to the better fit between the phenolic compound the CD cavity, since γCD had the biggest cavity [144]. A similar work used the native αCD and βCD and 2 derivatives (HPβCD and DMβCD) to increase the concentration of resveratrol on solution and it stability. It was observed that the IC with native CD was, only, capable of complex with part of the resveratrol molecule and that the HPβCD offered a cavity with a better fit to the bioactive molecule [148].

The biological properties of resveratrol (anti-oxidant and anticarcinogenic) were also enhanced by its encapsulation. For instances, Lu et al [146] and Li et al [147] used βCD and HPβCD as resveratrol carrier agents and described the betterment of the scavenging capacity of the IC, the inhibition of the lipid peroxidation activity and the cytotoxicity to cancer cells without harming the healthy ones [147]. The results obtained on the two works support the notion that the CD derivative form a stronger IC with this stilbene (K βCD 1815M^{-1} and K HPβCD 6778M^{-1}), related to the easier access of the resveratrol to the HPβCD cavity, due to enlargement of the cavity opening and the despairing of the intramolecular hydrogen bond network [146, 147]. Additionally, the employment of HPβCD for resveratrol encapsulation, also, increases the photostability of this natural compound. The host position of the 'guest' molecule inside the CDs did increase the resistance to the degradation by UV radiation, without affecting its anti-oxidant properties. Therefore, the HPβCD/resveratrol ICs represents a powerful candidate for the protection of skin against oxidative stressing episodes [122]. Furthermore, resveratrol concentration on aqueous environment was improved by the complexation with βCD and G_2βCD and, consequently, it anti-oxidant capacity. Besides the solubilisation, both CDs were capable of protect the phenolic compound from rapid oxidation, by entrapping it inside their cavities, with similar stability (K βCD 4317 M^{-1} and K G_2βCD 5130 M^{-1}) [184].

The curcumin is a natural phenolic with antitumour activity and effective against HIV-infection, cystic fibrosis and immunomodulation agent, but it low stability at acid environment and physiological pH and photosensibility limited the use of this coumarin as pharmaceutical agent [185]. Therefore, its encapsulation by the 3 native CDs was described by Patro et al [134]. They were able to improve the solubility and oral availability with all the 3 ICs. However, the α-CD was the one that showed higher K (1124 M^{-1}). Lopez-Tobar et al [133], also, applied βCD and γCD as curcumin drug carriers. The large cavity of γCD was more efficient in the IC formation. Moreover, both CDs were able to form 2:1 IC with this phenolic compound and the molecular interaction proposed was that the aromatic rings and the hydrogen bonds were involved and a change occurs from the curcumin planar ketoenol form to non-planar difeto. The chemical stability and bioavailability may be upgraded by this conformational alteration [133], The same stoichiometry of the IC βCD/curcumin was observed by Tang and co-workers [186] and Rahman et al [127]. In both situations, the solubility of curcumin was described as well as the same molecular interaction, referred above. Dandawate et al [132] used a synthetic form of curcumin but accomplished the same stoichiometry and solubility. In this work, the anticarcinogenic, systemic

bioavailability and tissue distribution of the IC βCD/synthetic curcumin were compared with the synthetic curcumin alone and concluded that they have been improved by the encapsulation [132].

CD derivatives were also tested as drug carriers of curcumin, in order to overcome the difficulties of its application as anticarcinogenic agent. For instance, 2 molecules of HPβCD formed stable IC (K 5000 to 62000 M^{-1}) with 1 molecule of curcumin [128, 130, 187]. The transdermal capacity of curcumin was raised by the complexation with HPβCD, as well as the decrease of skin irritation [128]. Besides, HPβCD, HPαCD and HPγCD were used with the same goal. Mohan et al [130] compared the encapsulation of the 3 CD derivatives and reported that the encapsulation may occur both in 1:1 and 2:1 stoichiometry, and the HPγCD has a better complexion capacity. This IC (HPγCD/curcumin) was capable of reducing cell proliferation and increase the apoptosis of cancer cells by interfering in the protein production [129]. Tonnesen et al [135] reported the encapsulation of curcumin with several CD derivatives, namely HPαCD, RMβCD, HPβCD, SBEβCD, HTAβCD and HPγCD. The greater positive change on the stability and solubility of curcumin was achieved by the complexation with the RMβCD and HPγCD, probably due do to the more hydrophobic environment found inside the cavity of the first CD and large cavity of the second, leading to a better accommodation of the bioactive molecule [135]. The increase of the curcumin resistance to hydrolysis under alkaline environments when encapsulated with these CDs, was also proved [135].

The application of CDs as carriers of non-flavonoids is a viable choice to protect them from degradation by environment factures, such as UV-light, pH, temperature and oxidation, and also to improve their solubility, factors that contribute to increase the biological properties of these natural active molecules. However, because this group of molecules is a bit diverse, it is not possible to generalize the molecular mechanisms of interaction between CDs and non-flavonoids and the efficiency of the encapsulation depends, essentially, on the size of the CD cavity.

Conclusion

CDs are capable of forming inclusion complexes with a wide range of bioactive molecules and, also, with polymers. CDs can act, simultaneously, as therapeutic agent carriers and as enhancers favourable hydrogel properties in biosystems.

Hydrogels provide major benefits for wound-dressing applications since they can retain a moist environment (crucial for wound healing purposes), and are biocompatible. However, with regard to drug delivery applications, the trapping of low-to-moderately hydrophobic bioactive molecules is not very efficient, and their release is rapid and non-linear with time.

The use of bioactive molecules from plants has gained a substantial interest during the last decade for food, cosmetic and pharmaceutical applications. Polyphenolic agents derived from plant sources have aroused much interest, especially in view of their anti-oxidant activity and bactericidal and fungicidal actions. Concerning the pharmaceutical applications of these plant-derived bioactive molecules, the current problems are related to the protection of their properties from environmental factors, with their solubility in water and biofluids, and their bioavailability.

CDs have a relatively unique capacity of improving solubility of bioactive polyphenolic agents in aqueous systems; protect them from elevated temperatures, pH values, light or the moisture-induced degradations phenomena which serve to increase their bioavailability. Furthermore, the use of substituted cyclodextrins has been found to improve the physicochemical properties of these bioactive molecules. In fact, in the case of flavonoids the CDs' derivatives are the better choice to achieve an efficient complexation. Otherwise, the selection of the better CD for encapsulated non-flavoinoids molecules needs to be based on the dimensions of the molecule and the CD's cavity. Nerveless, the formation of inclusion complexes between CDs and plant polyphenolics serves as a promising pathway for the development of pharmaceutical products friendlier to the user.

Therefore, cyclodextrin-based hydrogels may provide a solution to the achievement of a wound dressing material capable of maintaining appropriate conditions for wound healing, in addition to improving the healing process itself, via the release of bioactive molecules.

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Chapter 2

Antibacterial Potential of North-Eastern Portugal Wild Plant Extracts and Respective Phenolic Compounds

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Pinho E, Ferreira I, Barros L, Carvalho A, Soares GMB, Henriques M. Antibacterial potential of North-eastern Portugal wild plant extracts and respective phenolic compounds. BioMed Research International (Accepted).

Abstract

The present work aims to assess the antibacterial potential of phenolic extracts, recovered from North East Portuguese plants, and of their phenolic compounds (ellagic, caffeic and gallic acids, quercetin, kaempferol and rutin), against bacteria commonly found on skin infections. The disk diffusion and the susceptibility assays were used to identify the most active extracts and phenolic compounds. The effect of selected phenolic extracts and compounds on animal cells was assessed by determination of cellular metabolic activity. Gallic acid had a higher activity, against gram positive (*S. epidermidis and S. aureus*) and negative bacteria (*K. pneumoniae*) at lower concentrations, when compared with other compounds. The caffeic acid, also, showed good antibacterial activity against the 3 bacteria used. At the same concentration, gallic acid inhibited the growth of the 3 bacteria without causing harm to the animal cells. Nevertheless, gallic and caffeic acid showed a promising applicability as antibacterial agents for the treatment of infected wounds.

Keywords: caffeic acid, fibroblasts, gallic acid, phenolic compounds, skin and soft infections, *S. aureus*.

Introduction

Skin, the largest human organ, works as a mechanical barrier against environment hazards and is, also, responsible for self-healing, immune surveillance, sensor detection, thermoregulation and fluid homeostasis [1, 2]. Injuries, caused by extreme temperature, trauma, chronic ulcerations, pressure or venous stasis, promotes disruption of skin integrity allowing the deposition and colonisation of the injury tissue by a wide range of bacteria [3]. Skin and soft tissues infections are, typically, associated to staphylococci or streptococci, but virtually any microorganism may induce tissue inflammation and immune response [4, 5]. The severity of these infections may range from self-limit superficial infections to life-threatening diseases. The most common treatment is the use of broad-spectrum antibiotics. Though, the indiscriminate use of this kind of drugs affects the normal skin flora and may result in multi-resistant strains [6]. In order to overcome this issue it is critical to identify new antimicrobial agents.

Plants are a viable, unlimited source of bioactive molecules, including antimicrobial agents which protect them from microorganism, insects and predators [7–11]. Phenolic compounds belong to these bioactive molecules group; their pharmaceutical abilities and benefits for human health have been demonstrated in several published studies [7, 8]. Anti-inflammatory, anti-oxidant and antimicrobial are some of the properties attributed to those molecules [7, 8].

The north-eastern region of Portugal, Trás-os-Montes, gathers a wide range of wild plants used on folk pharmacopeia and traditional cuisine. Several ethnobotanical surveys conducted in this region by Mountain Research Centre (CIMO) - ESA, Polytechnic Institute of Bragança selected some of wild plants as potential source of natural antimicrobial agents.

The present work aims to select phenolic compounds, identified on extracts of selected wild plants from north-eastern region of Portugal, to be applied as antibacterial agents on the treatment of infected wounds. The antibacterial activity of 8 phenolic extracts and 6 phenolic compounds was tested against *Staphylococcus epidermidis*, *Staphylococcus aureus* and *Klebsiella pneumoniae*, usually, isolated from skin and soft tissue infections. The influence of the most effective phenolic compounds on human fibroblasts was, also, evaluated.

Material and methods

Phenolic Extracts and Compounds

The plant samples and their phenolic extracts were obtained as described by Barros et al: *Asparagus acutifolius* (shoots)- Aa and *Bryonia dioica* (young stems) Bd [12], *Cytisus multiflorus* (flowers)- Cm and *Sambucus nigra* (flowers)- Sn [13], *Rosa micrantha* (flowers)– Rm, *Filipendula ulmaria* (inflorescences)– Fu and *Castanea sativa* (upright catkins during anthesis)- Cs [14] and *Cistus ladanifer* (leaves)- Cl [15]. The phenolic characterization of the extracts is, also, described on the publications mentioned above. Six different phenolic compounds, recovered from those plants, were pointed out as the main ones: 3 phenolic acids (caffeic, ellagic and gallic acids) and 3 flavonoids (kaempferol, quercetin and rutin) [12–15].

Strains and Growth Conditions

The antibacterial activity of the phenolic extracts and compounds was tested against 3 bacteria: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296). The bacteria were grown in tryptic soy agar (TSA, Merck, Germany) for 24 h at 37 °C. The cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 18 h at 37 °C, under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to $1x10^6$ cells.mL^{-1}, via absorbance readings and the corresponding calibration curves.

Disk Diffusion Assay

The antibacterial activity of the extracts or compounds against the 3 bacteria was assessed, first, by the Disc diffusion method described by the National Committee for Clinical Laboratory Standards (NCCLS), M2-A8 document [16], with some modifications. The TSA was the nutritive media used. Afterwards, 200 µL of each inoculum ($1x10^6$ cells.mL^{-1}) was spread on the solid media plates (90 mm Petri dishes). Sterile filter paper disks ("Blanck Discs", Liofilchem, Roseto, Italy, 6 mm in diameter) were placed over the petri dish and impregnated with 20 µL of each extract (200 mg.mL^{-1}) or compound (5 mg.mL^{-1}). The plates were incubated at 37 °C for 24 h. Thereafter, the size of the halo from the inhibition growth was measured.

Susceptibility Assay: Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC)

MIC and MBC were obtained according to the method described by Wiegand [17], an adaptation of the standard methods published by Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST) [18], using the broth microdilution procedure. Thus, a work solution of 20 $mg.mL^{-1}$ of each extract and 10 $mg.mL^{-1}$ of each compound were prepared in sterile distilled water. The 96 well plate (Orange Scientific, Braine-l' Alleud, Belgium) were prepared by adding 100 µL of a solution of each extract/compound to a final concentration of 10 $mg.mL^{-1}$ / 5 $mg.mL^{-1}$ to the first well. Then serial dilutions (1:10) were made with MHB (Mueller–Hinton broth, Merck, Germany) in the other wells. The extracts concentration tested ranged between 0.02 and 10 $mg.mL^{-1}$ and the phenolic compounds from 0.01 to 5 $mg.mL^{-1}$. At each well, 50 µL each bacterium were added (*S. epidermidis, S. aureus and K. pneumoniae*). Drug-free and bacteria controls were also included. The plates were incubated for 24 h at 37 °C.

The MIC value was evaluated by the observation of the concentration that did not show any growth, by contrast with the bacteria control. The MBC, number of viable cells, was assessed by determination of the number of colony forming units (CFUs). The CFUs were measured by plating 10 µL of cell suspension from each well onto TSA, and incubated for 24 h at 37 °C.

The procedure was made in triplicate for each extract, compound and bacteria combination, in, at least, 3 independent assays.

Cytotoxicity Determination

Fibroblast 3T3 (CCL 163) from American Type Culture Collection was used in this study. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10 % of foetal bovine serum and 1 % penicillin/ streptomycin at 37 °C, 5% CO_2. After achieving the confluence, cells were passed at the density of 1×10^5 $cells.mL^{-1}$, using trypsin.

To assess the effect of the extracts or compounds on the cellular viability, the cells were seeded at the density of 5×10^5 $cells.mL^{-1}$ (24 well plate) in 1 mL of DMEM complete medium. After 24 h, the medium was replaced by 500 µL of fresh one, and 500 µL of extracts/compounds, at twofold of the desired concentrations dissolved in phosphate buffer solution (PBS). The plates

were incubated for 24 h at 37 ºC and 5 % CO_2. Afterwards, the medium was removed and a mixture of 20 µL of MTS [3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium] (Promega) and 980 µL of DMEM without phenol was added to each well. After 1h, the absorbance value was measured, at 490 nm, and the results were expressed as percentage of viable cells (%), using the number of cells grown on wells without compounds as controls.

The procedure was made in triplicate each extract/compound in, at least, 3 independent assays.

Results and Discussion

The emergence of multi-resistant strains of pathogenic and opportunistic bacteria is correlated with the widespread use of broad-spectrum antibiotics, for treatment of skin and soft tissue infections. Therefore, the search for new drugs and new sources of antibacterial agents is of outmost importance [19]. Natural sources, such as plants, have been explored and gained prominence, since they offer many advantages when compared to the synthetic ones. For instances, they show high levels of biocompatibility and availability, and low toxicity [7–10]. Currently, polyphenolics are one of the major group of interest in view of their anti-inflammatory, antimicrobial, antiviral and anti-oxidant properties [20].

Antimicrobial Activity of the Extracts

The antibacterial activity of phenolic extracts from medicinal Portuguese plants, and, also, from polyphenolic compounds, identified on those extracts, was assessed by both qualitative and quantitative methods. The disk diffusion assay is a qualitative method, which allows a first screening of the potential antibacterial agents. However, this method presents some issues regarding the active molecules ability to diffuse into the agar, and so, a quantitative method such as the MIC and MBC determination should be used in order to obtain more accurate results. The MIC is defined as the lowest concentration of the antibacterial agent that inhibits the visible bacteria growth, observed with unaided eye, and the MBC is the minimal concentration of the antibacterial agent required to destroy most of the viable bacteria (reduce at least 3 log of growth) for a given set of conditions [21]. It is important to refer that the extracts and compounds present some colouration, which may lead to some misleading of the MIC values. Therefore, the MBC determination is crucial for a complementary analysis of the antibacterial properties of the phenolic compounds [19].

A preliminary assay using phenolic extracts of Portuguese medicinal plants was made, in order to identify phenolic compounds from those extracts. The chemical characterization of the phenolic extracts was described in previous works [12–15]. Both qualitative and quantitative analysis was made to the antibacterial activity of 8 phenolic extracts. From those extracts, only 5 (Cs, Cl, Cm,

Fu and Rm) were capable to reduce the growth of the 3 bacteria used (Fig 2.1). These extracts were selected as the most promising ones and their MIC and MBC values were assessed.

As, the disk diffusion assay is based on the measurement of the growth inhibition halo, which is dependent on the antibacterial agent ability to diffuse trough agar, leading sometime mislead the results, quantitative complementary assays were also performed.

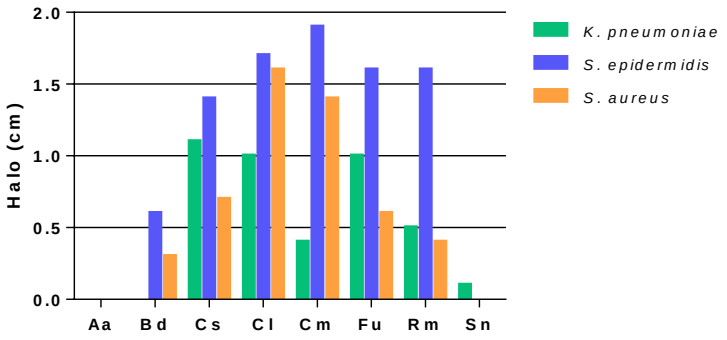

Fig 2.1 Values of the halo dimension (disk diffusion assay) for each extract (200 mg.mL^{-1}) for the 3 bacteria. The halo size was calculated by deducting the size of the disk (0.6 cm). Aa: *A. acutifolius,* Bd: *B. dioica,* CS: *C. sativa,* Cl*: C. ladanife,* Cm: *C. multiflorus,* Fu: *F. ulmaria,* Rm*: R. micrantha* and Sn*: S. nigra*.

The MIC and MBC values revelled that the Cs and Cl had a similar effect on the bacteria, being more effect against *K. pneumoniea* and *S. epidermidis* (MIC and MBC 0.63 mg.mL^{-1}, for both species) and less effect against S. aureus (MIC 1.25 and MBC 2.5 mg.mL^{-1}). The Fu and Rm extracts, also, showed comparable effect against the bacteria, namely, 2.5 mg.mL^{-1} was capable of reduce completely the 3 bacteria growth when exposed to these 2 extracts. Since, Cm phenolic extract had MIC and MBC values higher than 10 mg.mL^{-1}, this extract was not used for further analysis.

Extracts Effect on Fibroblast Proliferation

Besides the antimicrobial activity of the extracts, the knowledge of their effect on the human cells is also crucial. Therefore, to predict the influence of the most promising phenolic extracts (Cs, Cl, Fu and Rm) on animal cells proliferation, a preliminary assay of cytotoxicity was made.

It was observed that the higher concentrations of the extracts (10 and 5 $mg.mL^{-1}$) reduced the number of viable cells, when compared with the fibroblast growth without extracts. The phenolic extracts in lower concentration (1.25 $mg.mL^{-1}$) had the opposite effect on the human cell line improving their growth and adhesion.

A variation on the impact of the phenolic extracts was detected when using 2.5 $mg.mL^{-1}$. The Cs and Cl are still toxic at this concentration, but Rm improved cell growth and the Fu phenolic extract show similar number of viable cells, in relation to the control. Thus, the results regarding the effect of Cl extract on animal cells in vitro are in accordance with Barrajón-Catalán et al [22] and Andrade et al [23]. Both studies show that Cl extract inhibit the proliferation of the cells when concentrations higher than 1.25 $mg.mL^{-1}$ were used.

Regarding to the cytotoxicity of the Fu extract, it improved the cell growth at 1.25 $mg.mL^{-1}$. However, others authors [24–26] results suggest that this extract inhibit the growth of animal cells at lower concentration (between 0.2 a 0.1 $mg.mL^{-1}$). The cell lines (B16 mouse melanoma [24, 25] or human lymphoblastoid cells [26]) and the method for quantify the viable cells (tripan blue coloration) used were different from our conditions, justifying the divergence of results.

To authors' knowledge, the effect of the Cs and Rm phenolic extracts on animal cells has been not described until know. The Rm extract improved the 3T3 fibroblast growth at 2.5 $mg.mL^{-1}$ and CS at 1.25 $mg.mL^{-1}$. Furthermore, the Rm and FU extracts can be safely used as antibacterial agents without causing any harm to the human cells.

Antimicrobial Activity of the Phenolic Compounds

Six phenolic compounds identified in the extracts of Cl (ellagic acid, kaempferol and gallic acid), Fu (caffeic acid, kaempferol, rutin and gallic acid), Cs (gallic acid and rutin) and Rm (kaempferol) were selected for further analysis. Those compounds are all polyphenolics and can be placed into two groups: (1) phenolic acids and (2) flavonoids. Caffeic, gallic and ellagic acid belong to the first group and the remain compounds (kaempferol, quercetin and rutin) fit into the flavonoids group.

The disk diffusion assay of the phenolic compounds (Fig 2.2) demonstrated that they can inhibit the growth of *S. epidermidis*, being the gallic acid the most efficient and caffeic acid, rutin, and quercetin the least. The compounds present a similar halo size against *S. aureus*, with the

exception of ellagic acid that did not change the bacteria growth. Gallic acid and caffeic acid were the only phenolic compounds tested capable of inhibit the gram negative bacteria (*K. pneumoniae*) growth. Due to the different and interesting results of the compounds in the diffusion assay, it was decided to determine the MIC and MBC of all of them (Table 2.1).

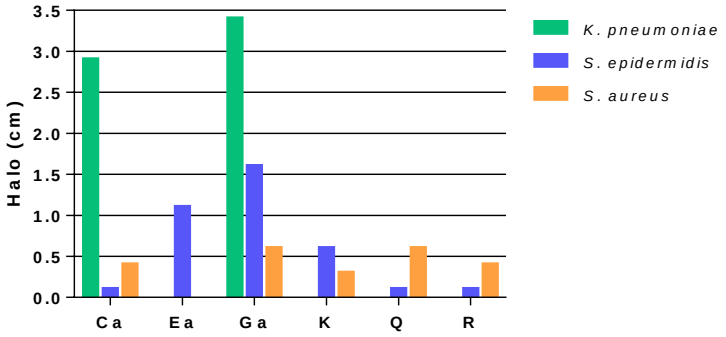

Fig 2.2 Values of the halo dimension (disk diffusion assay) for each extract (5 mg.mL^{-1}) for the 3 bacteria. The halo size was calculated by deducting the size of the disk (0.6 cm). Ca: Caffeic acid; Ea: ellagic acid; Ga:gallic acid; K: kaempferol; Q: quercetin; and R:rutin.

Flavonoids are a group of polyphenolic molecules from plant source with many biological properties already studied [27, 28]. The flavonoids antibacterial capacity is based on their ability to complex with extracellular and soluble proteins, and to destroy the bacteria cell wall by interact with essential enzymes, responsible for maintained the stability of this structure [7, 19]. However, in the conditions tested the flavonoids selected (kaempferol, quercetin, and rutin) had no effect on the bacteria growth for concentrations under 5 mg.mL^{-1}.

Our results, similarity to Penna et al [29], suggest that kaempferol has no activity under 5 mg.mL^{-1} against *S. aureus*. Additionally, Fattouch et al [30] showed that kaempferol presented activity only at a concentration of 10 mg.mL^{-1}. Regarding quercetin antimicrobial activity, El-Gammal et al [31] described that its MIC for *S. aureus* was 37 µg.mL^{-1}. Fattouch et al [30] achieved a MIC and MBC for quercetin of 10 mg.mL^{-1} for the same bacteria, which corroborates our results. In this case, the differences among results can be justified by the methods used. For instance, Fattouch et al [30] used the microdilution method, the same procedure used in this work, however El-Gammal et al [31] used a method dependent on the diffusion capacity of the compounds, which justify the differences on the MIC of the quercetin. In the case of rutin, some authors described

S. aureus was 0.125 mg.mL^{-1}, and Thiem and Goslinska [36], besides MIC, also, determined the MCB of this phenolic acid against the same bacteria and reported the values of 0.63 and 2.5 mg.mL^{-1}, respectively.

In literature, the gallic acid and the caffeic acid showed the same antibacterial mechanism, related to their similar structure (Table 2.1). These phenolic acids disrupted the bacteria cell by hyperacidification of the plasma membrane via proton donation and acidification of the intracellular cytosolic; this low pH can inhibit the enzyme H^+-ATPase, necessary for the ATP production [37–39]. Our results demonstrated that the gallic acid and the caffeic acid had effect against the 3 bacteria tested. However, the first phenolic was active against both gram positive and negative bacteria with concentration on the range of µg.mL^{-1} and the caffeic acid activity was only detected when concentrations between 0.63 and 5 mg.mL^{-1} were used. Moreover, the *K. pneumoniae* was the most resilient bacteria to this compound (Table 2.1).

Most of the published works regarding the antibacterial activity of gallic and caffeic acids use the disk diffusion assay method, without any quantitative method for complementary analysis [37, 40–42], which may lead to misleading results related to the capacity of these molecules to diffused into the solid medium. Regarding the caffeic acid activity, Vaquero and Kwon reported formation of halo when in contact with gram positive and negative bacteria for 50 mg.mL^{-1} [37, 42]. Larrainzar et al [40] obtained a MIC value for *S. aureus* of 561 µg.mL^{-1}of gallic acid and Binutu et al [43] had a MIC of 250 µg.mL^{-1}, 10 and 7 times higher, respectively, than the value attained in this work despite of the method used was the same. The differences of the MIC value may rely on lower temperature (lower metabolic rate leading lower uptake at 35 °C when compared with 37 °C) used for the growth of *S. aureus* by Larrainzar et al [40] or in a misleading caused by the colouration of the gallic acid [19].

The differences find on the results published may be due to the diverse methods applied in each work to assess the MIC and MBC and/or interferences on the MIC and MBC procedure, such as, variations on the volume and concentration inoculum, source of the polyphenolic (natural or commercial), salts formation and precipitation leading to misleading results [19].

Effect of Gallic and Caffeic acid on 3T3 Fibroblast Growth and Adhesion

Besides the antibacterial activity of natural molecules, the knowledge of their effect on the human cells is, also, crucial. Therefore, to predict the effects of the most promising phenolic compounds (gallic and caffeic acid) on animal cells, a preliminary assay of cell viability was made (Fig 2.3).

The gallic acid showed no toxicity to the fibroblast when concentrations between 0.01 and 0.1 $mg.mL^{-1}$ were used (Fig 2.3 a). In fact, the lowest concentration induced an increase on the number of viable cells measured. However, for concentrations above 0.1 $mg.mL^{-1}$, gallic acid became toxic.

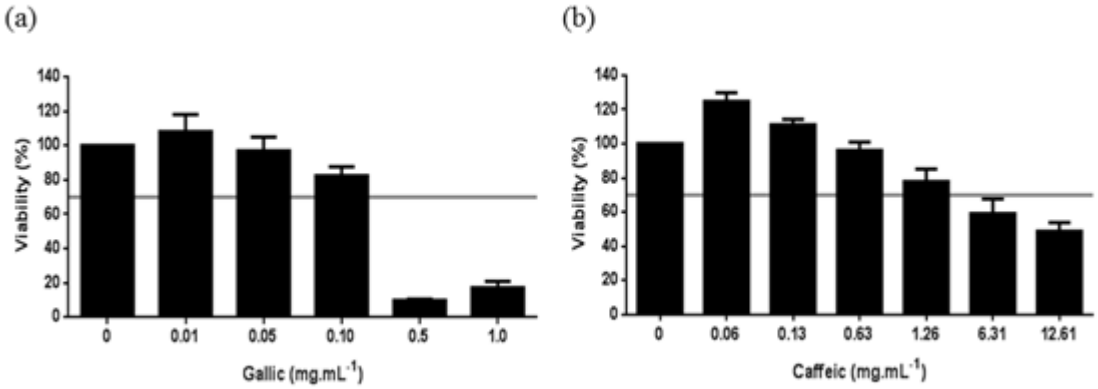

Fig 2.3 The viability of cells after 24 h of contact with gallic acid (a) and caffeic acid (b) dissolved in PBS, measured with an MTS assay. All data is expressed as mean + standard deviation (n = 9).The line indicate 70% of cell viability.

The effect of caffeic acid on the viability of cells was dose dependent. The caffeic had no significant influence on the cells growth for concentrations between 0.06 and 1.26 $mg.mL^{-1}$, but a reduction greater than 30% of viability was measured when 6.31 $mg.mL^{-1}$, or higher, were applied.

Both phenolic compounds have been described as potent anti-oxidant, and as consequence they exert some chemopreventives effects on animal cells [44, 45]. However, their anti-oxidant activity is based on oxidation-reduction reactions that are reversible and dependent on concentration. Therefore, these phenolics can act both as anti-oxidant and pro-oxidant, depending on the reaction conditions [44, 46]. This explains the fact that for higher concentrations gallic and caffeic acid induce major reduction on the cells viability. Additionally, gallic acid was more toxic, since that less than 20% of cells were capable of surviving for the concentrations between 0.5 to

1 $mg.mL^{-1}$ (Fig 2.3 a). For the same range of the concentrations, the cells tolerated the caffeic acid (Fig 2.3 b). Our findings corroborate the fact that the predisposition to act as pro-oxidant is directly proportional to the number of hydroxyl groups (OH) in the molecule, gallic acid has 4 OH and caffeic only 3 (Table 2.1).

Nevertheless, gallic acid can be used as antibacterial agent against the bacteria tested without cause any damage to the animal cells, since its MBC was 0.04 $mg.mL^{-1}$ and, at this range of concentrations, the percentage of viable cells measured was higher than 70%, above this limit the compounds are safety for humans based on the ISO 10993-5:2006. In the case of caffeic acid, the concentration capable of destroy all the bacteria was, also, toxic to the fibroblast, which may suggest that the mechanism involved on the antibacterial action is the same of the on that cause damage on animal cells.

Conclusion

The results presented in this work highlight the potential of phenolic extracts from wild Northeast Portuguese plants as antibacterial agents, as well as some of their phenolic compounds.

Overall, extracts from *Cistus ladanifer, Cytisus multiflorus, Castanea sativa, Filipendula ulmaria* and *Rosa micrantha* were capable to inhibit the growth of the 3 bacteria commonly isolated from skin and soft tissue infections. Moreover, *Cistus ladanifer, Castanea sativa, Filipendula ulmaria* and *Rosa micrantha* revealed promising antibacterial effects against *K. pneumoniae, S. epidermidis* and *S. aureus*, in concentrations between 0.625 and 2.5 $mg.mL^{-1}$.

From those extracts, 6 phenolic compounds were selected. The flavonoids (kaempferol. quercetin and rutin) were capable of inducing halo formation on gram positive bacteria. However, the quantitative assay of the flavonoids demonstrated that they were not active for concentrations below 5 $mg.mL^{-1}$. Regarding the phenolic acids, the ellagic acid was only active against *S. epidermidis*, but gallic and caffeic acids showed good antibacterial activity against the 3 bacteria at low concentrations. Thus, their effect on fibroblast proliferation was assessed, and revealed that caffeic acid has dose-response cytotoxicity and can be considered safe for concentrations less than 6.31 $mg.mL^{-1}$. Regarding gallic acid, at lowest concentration it promoted the proliferation of fibroblast, but for concentrations above 0.1 $mg.mL^{-1}$ it became toxic. Nevertheless, conjugating the antibacterial and cytotoxicity results, it could be pointed off that gallic acid can be used safely and presenting antibacterial activity against the 3 bacteria.

The present work pretends to be a starting point to the use of phenolic compounds from North Eastern Portugal Plants on the treatment of infected wounds, instead of large-spectrum antibiotics. Moreover, gallic and caffeic acid appears to be suitable for incorporation on wound dressings to avoid tissue infections.

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Chapter 3

Phenolic Acids Encapsulation by Cyclodextrins

Chapter 3.1

Cyclodextrins modulation of gallic acid *in vitro* antibacterial activity

THE PRESENT CHAPTER WAS ADAPTED FROM THE FOLLOWING PAPER

Pinho E, Soares GMB, Henriques M.

Cyclodextrins modulation of gallic acid *in vitro* antibacterial activity

Journal of Inclusion Phenomena and Macrocyclic Chemistry (submitted)

Abstract

The substitution of large spectrum antibiotics for natural bioactive molecules (especially polyphenolics) for the treatment of wound infections has gain importance on the pharmaceutical industry. However, the use of those molecules depends on their stability in environmental stress and ability to reach the action site without losing biological properties. The application of cyclodextrins as vehicle for polyphenolics protection has been reported and appears to enhance the bioactive molecules properties.

Therefore, the inclusion of the poor stable antibacterial agent, gallic acid, was investigated in β-cyclodextrin, hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin. Encapsulation by β-cyclodextrin was confirmed for pH 3 and 5, with similar stability parameters. The hydroxypropyl-β-cyclodextrin and methyl-β-cyclodextrin interactions with gallic acid were only confirmed at pH 3. Among the cyclodextrins, the better parameters of gallic acid encapsulation were obtained for hydroxypropyl-β-cyclodextrin, followed by β-cyclodextrin and methyl-β-cyclodextrin. The effect of CD encapsulation on the gallic acid antibacterial activity was, also, analysed. The antibacterial activity of the inclusion complexes was confirmed here, for the first time.

Therefore, the encapsulation of gallic acid by hydroxypropyl-β-cyclodextrin seems to be a viable option for the treatment of skin and soft tissue infections, since this inclusion complex has good stability and antibacterial activity.

Keywords: Antibacterial activity, Benesi-Hildebrand equation, β-cyclodextrin, gallic acid, hydroxypropyl-β-cyclodextrin, methyl-β-cyclodextrin.

Introduction

In the last years, the application of cyclodextrins (CD) as functional carriers in the pharmaceutical industry has increased. CDs are cyclic oligosaccharides with a truncate cone shape, their hydroxyl groups (OH) are oriented to the outer molecular surface and the hydrophobic groups turned to the cavity. This creates an micro-heterogeneous environment, which allow the formation of inclusion complex (IC) with a wide range of molecules, from straight or branch aliphatic chains to polar compounds, changing their chemical, physical or biological properties [1, 2].

The IC stability is, extremely, dependent on the three-dimensional fit between CD' cavity and the "guest" molecule and, on the specific local interactions between the CDs' surface groups and the "guest" [3]. The complex stability relies on hydrophobic forces, hydrogen bonds, van der Waals interactions, and on other factors like the release of ring strain and modifications in solvent surface tension. The combinations of these factors render the IC to a more stable energetically state [4, 5]. Thermodynamic factors, like enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG), can be used as parameters for the complexation process evaluation, since the temperature influences the selectivity of the interaction between CD and the bioactive molecule [6]. In the case of organic compounds as guests, additional factors, such as pH and solvents, seems to play a major role on the IC formation [7].

In nature, CDs exists as α, β and γ differing on the cavity volume and diameter, being βCD the most used on the industry due to its capacity to encapsulate with a wide range of molecules. This native cyclodextrin has been subjected to chemical alterations on its OH, in order to enhance the physiochemical properties [7]. For instances, the 2-hydroxypropyl-β-cyclodextrin (HPβCD) and methyl-β-cyclodextrin (MβCD) are more hydrophilic than the βCD. Moreover, these derivatives presents higher solubility than the βCD (500 and 750 $g.L^{-1}$ at room temperature compared to 18 $g.L^{-1}$ for βCD), which enhance the complexation with poor water soluble molecules [8].

Wound infection has been one of the major causes of delay on the healing process or even on the scar development. Those infections, often associated to *Staphylococcus* and *Klebsiela* species, may range from superficial infections to life-threatening in compromised patients. Broad-spectrum antibiotics has been, indiscriminately, used for the treatment of skin and soft tissue infections, changing the normal skin flora and leading to multi-resistant strains [9]. Therefore, the

demand for new antibacterial agents has increased in the last years and polyphenolics has been one of the major group of compounds study for that propose [7].

Gallic acid, also known as 3,4,5-trihydroxybenzoic acid, is a simple phenolic acid common found in plants, with several biological activities. For instance, gallic acid has been described as anti-oxidant, anti-inflammatory, anticarcinogenic and antimicrobial [10–13]. Although, as others polyphenolics, gallic acid as reduced pharmacological applicability due to it lower water solubility [14, 15] and sensitivity to environmental stress (pH, light, temperature), factors that cause poor bioavailability [16–18].Thus, to maintain the gallic structural integrity and allow it to achieve the physiological targets, without losing any activity, an encapsulation device is necessary.

Several authors have reported the phenolic acids encapsulation by CDs, being the native CD the most used, as well as the hydroxycinnamic acids (caffeic, chlorogenic, caffeic and rosmarinic acid) [8, 19–22]. They all report the IC formation with 1:1 stoichiometry, but the phenolic orientation within the CD depends on the phenolic as well as the IC stability. Moreover, the utilization of CD derivatives (HPβCD and MβCD) was reported by Çelik et al [8]. The IC obtained with rosmaniric acid had better stability than the native CD, suggesting that the substitution facilitates the encapsulation [8].

Therefore, this work aims at evaluating the effect of pH on the stability of the inclusion complex formation between gallic acid and 3 CDs (βCD, HPβCD and MβCD). Additionally, the influence of gallic acids encapsulation on it antibacterial activity was assessed by the first time.

Material and methods

Material

Gallic acid (3,4,5-trihydroxybenzoic acid) was purchased from Merck, β-cyclodextrin (βCD, 1135 $g.mol^{-1}$) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, 1309 $g.mol^{-1}$) were acquired from AppliChem, and the methyl-β-cyclodextrin (MβCD, 1310 $g.mol^{-1}$) was obtained from Wacker. Gallic acid stock solution (2 or 10 mM) was prepared in methanol (MeOH) and kept for 30 min in an ultrasonic bath. Stock solutions for each CD ($4x10^{-2}$ M) were prepared in distilled water. The βCD solution was maintained at 50 °C and 200 rpm, in order to improve its solubility in water.

Buffer and pH effect

Gallic acid solutions ($1x10^{-5}$ M, 2% MeOH) were prepared in two different buffers (H_3PO_4/NaOH and KH_2PO_4/K_2HPO_4) at pH 3, 5, 7 and 8. The buffers were prepared as follows: the desired pH (3, 5, 7 and 8) was made by mixing proper amounts of H_3PO_4 ($1x10^{-2}$ M, pH 2.05) and NaOH (1 M, pH 14) for H_3PO_4/NaOH buffer and K_2HPO_4 ($5x10^{-3}$ M, pH 8.02) and KH_2PO_4 ($5x10^{-2}$ M, pH 2.89) for the buffer KH_2PO_4/K_2HPO_4. The solutions were maintained 30 min in ultrasonic bath to insure the total solubilisation of gallic acid. The UV-Vis absorbance spectrum of gallic acid was recorded between 200-360 nm, for each condition.

Inclusion Complex Preparation

In order to determine the stoichiometry and stability constants of the inclusions complexes between gallic acid and the 3 CDs, solutions with different concentrations of each CD (between 0 and $6x10^{-3}$ M) were added at the same concentration of gallic acid ($1x10^{-5}$ M), for each pH (buffer H_3PO_4/NaOH). The solutions were kept 30 min at ultrasounds bath, after they were maintained 24 h at 25 °C, and 50 rpm on dark. Samples of each solution were taken for absorbance measurements.

All the absorption spectra were measured the range 200–360 nm and recorded on a Jasco V560 spectrometer, using a 1cm quartz cuvette.

Stoichiometry, Stability Constant and Thermodynamic Parameters Calculation

The stoichiometry and stability constant (K) of each complex at the different pH were assessed based on the modified Benesi-Hildebrand equation [23] (equations 3.1.1 and 3.1.2).

(3.1.1) $\frac{1}{A-A_0} = \frac{1}{A'-A_0} + \frac{1}{K(A'-A_0)[CD]_0}$

(3.1.2) $\frac{1}{A-A_0} = \frac{1}{A'-A_0} + \frac{1}{K(A'-A_0)[CD]_0^2}$

[CD]₀ Cyclodextrin initial concentration

A Absorbance intensities in the presence of cyclodextrin

A₀ Absorbance intensities without cyclodextrin

A' Limiting intensity of absorption

A double reciprocal Benesi-Heldebrand plot was drawn using both equations, i.e. $\left(\frac{1}{A-A_0}\ vs\ \frac{1}{[CD]}\right)$ or $\left(\frac{1}{A-A_0}\ vs\ \frac{1}{[CD]^2}\right)$. The better fit (higher r^2) of the Benesi-Heldebrand plots was used to identify the ICs stoichiometry. The equations were used to define the inclusion complexes stoichiometry: equation (3.1.1) represents a 1:1 complex and the equation (3.1.2) 1:2 complex. The K was obtained from the slope of the graphic.

The Gibbs free energy (ΔG) was calculated using the K and equation 3.1.3.

(3.1.3) $\Delta G = -RTln(K)$

ΔG Gibbs free energy

R gas constant

T temperature (Kelvin)

K stability constant

BACTERIAL SUSCEPTIBILITY TO GALLIC ACID INCLUSION COMPLEX

Bacterial Suspension

The antibacterial activity of the gallic acid on buffer H_3PO_4/NaOH (selected buffer), at different pH values, as well as the IC activity, were tested against the bacteria: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296). The bacteria were grown in tryptic soy agar (TSA, Merck, Germany) for 24 h at 37 °C. The cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 24 h at 37 °C,

under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to $1x10^{6}$ cells.mL^{-1}, via absorbance readings and determined with the corresponding calibration curve.

Susceptibility Assay of Gallic acid

The minimal bactericidal concentration (MBC) was obtained according to the method described by Wiegand [24], an adaptation of the standard methods published by Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000), using the broth microdilution procedure. Thus, working solutions of $3.74x10^{-3}$ M of gallic acid were prepared at pH 3, 5, 7 and 8 (buffer H_3PO_4/NaOH). Serial dilutions of these solutions were made with MHB (Mueller–Hinton broth, Merck, Germany) to a final volume of 50 µL. Afterwards, 50 µL of each bacterium suspension were added to a final concentration of $5x10^{5}$ cell.mL^{-1}. Gallic acid- and bacteria-free controls were also included. The plates were incubated for 24 h at 37˚C. The number of viable cells, was assessed by determination of the number of colony forming units (CFUs), by plating 10 µL of cell suspension from each well onto TSA, and incubated for 18 h at 37 °C.

The procedure was made in triplicate for each pH and bacteria combination in, at least, 3 independent assays.

Antibacterial Activity of Inclusion Complexes

The ICs capacity to destroy the bacteria was also measured quantitatively. A volume of 50 µL of each complex (IC βCD/gallic acid and IC HPβCD/gallic acid) was added to 50 µL of $1x10^{6}$ cells.mL^{-1} of each bacterium, on 96 well plate. Bacteria and medium controls were also included. The plates were incubated for 24 h at 37 °C. The antimicrobial activity of each solution was assessed by determination of the number of CFUs, as described above. The procedure was made in triplicate for each bacterium, at least, in 3 independent assays. Solutions of buffer, gallic acid (1 mM), βCD (1 mM) and HPβCD (1mM) were also included, to ensure that none of these factors alone could have influence on the antibacterial activity of the ICs.

All the mathematical analysis was made using the Origin Pro software.

Results and Discussion

The biological properties of gallic acid remain active even at low concentrations [18], however this phenolic acid is quiet susceptible to degradation under environmental stress (pH, temperature, light and oxygen) [25]. Therefore, the gallic acid protection by encapsulation by CD was studied.

Influence of Buffer and pH on the Gallic acid Properties

The UV-Vis spectra of polyphenols reflects alterations of the electronic energy levels within the molecules, caused by electronic transitions between π-type molecular orbitals [26, 27]. The nature of the solvent, steric effects, formation of resonance forms, intra- and inter- molecular hydrogen-bonding, electron-donating and electron-withdrawing substituents on the benzene ring are factors responsible by alterations on polyphenols spectra [25].

Therefore, the influence of 2 different buffers on the UV-Vis spectra of gallic acid was analysed, as well as the effect of the pH for each buffer (Fig 3.1.1).

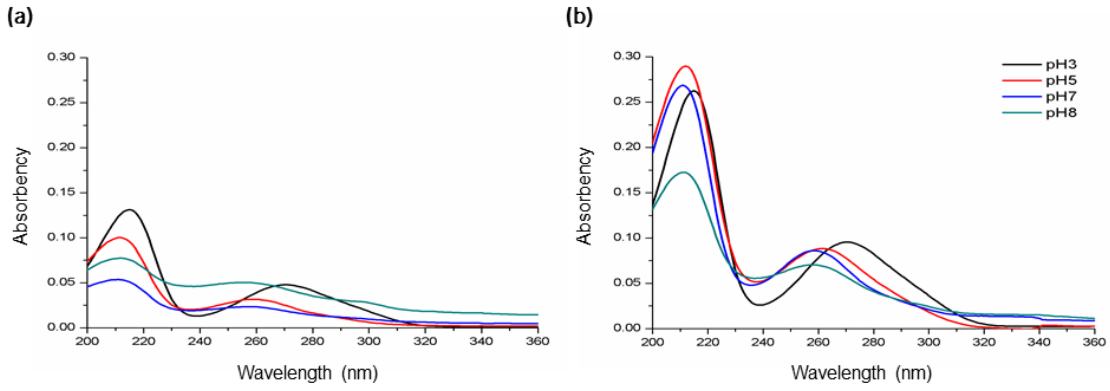

Fig 3.1.1 Absorbency spectra of gallic acid (1x10^{-5} M) dissolve in KH_2PO_4/K_2HPO_4 (a) and H_3PO_4/NaOH (b) buffers at pH3 (black), pH 5 (red), pH7 (blue) and pH8 (green).

The gallic acid UV-Vis spectra usually show 2 bands between 200 and 360, a b-band (lower wavelength) near 220 nm and a c-band (higher wavelength) near 270 nm. The buffer effect on the gallic acid spectra was notorious. The intensity of the 2 peaks, regardless of the pH, was

lower when the phenolic was dissolved in the KH_2PO_4/K_2HPO_4 buffer (Fig 3.1.1 a). At pH8, the peaks were not distinct, this peaks dissolution may be the result of a higher oxidation state of the phenolic compounds related to its unstable nature on basic environments, more notorious with this buffer [25, 28].

The H_3PO_4/NaOH buffer allowed higher intensity of the characteristics peaks, and the changes caused by pH were, also, more evident. The gallic acid spectra profile was the same for the pH range tested. However, an increase of pH induced a blue shift (a shift of λ_{max} towards shorter wavelengths) for both bands, being more notorious for the c-band (pH 3 273nm, pH 5 261 nm, and pH 7 and 8 258 nm) and, also, increase of intensity (hyperchromic effect). Therefore, the H_3PO_4/NaOH buffer was chosen for further work, since the effect of pH on the gallic acid UV-Vis spectra was more obvious when it was used.

As referred above, the pH is strongly linked to the stability of the polyphenols and affects their UV-Vis spectra [25]. Gallic acid has two ionisable moieties: (1) carboxylic group and (2) OH attached to the phenolic ring (Fig 3.1.2). The environmental pH will define the ionization state of gallic acid. The phenolic acid will assume a neutral form (Fig 3.1.2 a) at acid pH (<3.4), a carboxylic anionic between 4 and 7 and for basic pH appears as both carboxylic and hydroxyl anion [28, 29]. The gallic acid chemical alterations with the increase of pH have been attributed to the 3 hydroxyl groups (Fig 3.1.2 b). The formation of unstable quinones intermediates and other resonance forms has been linked to the reduction of intensity of c-band as the pH increase (Fig 3.1.1) [25]. Moreover, for basic pH (>7) gallic acid has been describe as unstable, suffering fast autooxidation, leading to the formation of degradation products [25, 28]. The presence of those products may justify the alterations observed on the spectra at pH 8, for both buffers.

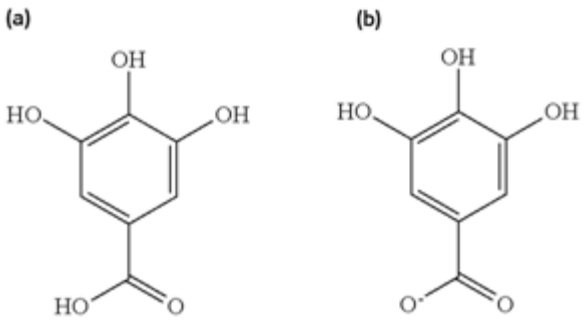

Fig 3.1.2 Chemical structure of neutral gallic acid (a) and gallic acid carboxylic anion (b).

The skin and soft tissues infections result from the colonization and proliferation of complex poly-microbial communities. The infections may be triggered by natural microflora, such as *Staphylococcus*, *Micrococcus* and *Corynebacteriumsp* or by not typical resident microflora gram negative bacteria (*Klebesiella, Capnocytophaga*, *Bartonella)*. The proliferation of pathogenic bacteria on the wound site has been related with the environmental pH [30, 31].

In addition, the gallic acid antibacterial ability has been related to its capacity to exchange protons with the bacteria and the environment, thus pH-dependent. The antibacterial mechanism of gallic acid relays on its affinity to the lipophilic membrane layer, which enable the phenolic transport and, consequently, cytoplasm acidification causing protein denaturation. The acidification of cell environment leading to variations on the potassium ions efflux, altering the electrical potential of the cell and improve it permeability. All these cascade of events takes to irreversible alterations on cell and death [32, 33].

Since the wound infection microflora depends on the environmental pH, as well as the gallic acid biological properties [25], the phenolic antibacterial activity at different pH was study (table 3.1.1). It was used 3 bacteria, usually, isolated from infected skin, two gram positive (*S. epidermidis* and *S. aureus*) and one gram negative (*K. pneumoniae*) [34].

Table 3.1.1 Minimal bactericidal concentration of gallic acid dissolved on H_3PO_4/NaOH (pH 3, 5, 7 and 8) against *K. pneumoniae, S. epidrmidis* and *S. aureus* ($5x10^5$ cel.mL^{-1})

MBC (mM)	*K. pneunmoniae*	*S. epidermidis*	*S. aureus*
pH 3	0.47	0.47	0.47
pH 5	0.23	0.23	0.47
pH 7	0.23	0.23	0.47
pH 8	0.12	0.23	0.47

Regarding the gram positive bacteria, the pH had no influence on the MBC obtained for *S. aureus* (0.47 mM) and MBC for *S. epidermidis* was 0.47 mM, at pH 3 and 0.24 mM for higher pH. It was described that *S. epidermids* growth is enhanced by acid environments [31]. Thus, the lower susceptibility of this bacterium at pH 3 may result from higher metabolic activity. This results, suggest that all forms of gallic acid (neutral and anionic) are activity against gram positive bacteria, on the conditions tested. A relation between the pH and the susceptibility of *K.*

pneumoniae to gallic acid was observed, the MBC obtained decreased with pH basification. The optimal growth pH for this gram negative locate near 5 and 6 [35], the pH used to access the bacterial susceptibility were beyond that pH range. Bacteria cells have mechanisms responsible for controlling the pH variation between the environment and cytoplasm. The maintenance of optimal cytoplasmic pH comprises a combination of strategies, such as cytoplasm buffering, adaptations on the membrane structure, active ion transportation and metabolic consumption of acids and bases [36]. However, in specific situations the pH homeostasis mechanisms can fail, leading to significant change on the variation pH and decrease the cell metabolism. One of those situations, may occur when the cell increase the uptake of weak acids to equilibrate the difference between external and internal pH. The weak acids can freely be transported through the bacteria membrane, causing protons release and consequent cytoplasm acidification [37].

In the present work, the addition of different pH buffers to the culture medium triggered those pH homeostasis mechanisms. Thus, the pH-dependent MBC obtained for *K. pneumoniae* may be a consequence of rising uptake of gallic acid (weak acid) to keep the pH homeostasis, which result on the accumulation of the organic acid on the cytoplasm, hyperacidification and, ultimately, cell death [37].

Impact of pH on the βCD/Gallic acid Interaction

The gallic acid c-band (near 270 nm) was selected for the analysis of the interactions with CD, once it is the most use for its characterization (HSDB -Hazardous Substances Data Bank). Fig 3.1.3 displays the effect of βCD concentration and pH on gallic acid UV-Vis absorbance spectra, collected after 24 h of complexation. Regardless the pH, as the CD concentration increase the λ_{max} intensity of gallic acid decreased. At higher pH (7 and 8) the alterations caused by βCD on the phenolic spectra were subtle, just a slight absorbance variation was detected (Fig 3.1.3).

The reduction of pH highlighted the effects of CD on the gallic acid spectra. At pH3 and 5, the spectra show an isosbestic point near 225 nm and hypsochromic effect (the λ_{max} shift to lower wavelength). The isosbestic point states the presence of gallic acid in free form and encapsulated. The hypochromic effect observed as CD concentration increase may indicate that gallic acid was totally embedded in the cavity [19]. Both alterations support the IC βCD/gallic acid formation at this pH range and that the groups involved on the complexation process are located near the chromophore [19]. Others authors reported similar effects of βCD on the gallic

acid spectra [15, 18, 29]; they also confirmed the encapsulation by SEM (scanning electron microscope), where the free gallic acid morphology was not detected after molecular inclusion [18].

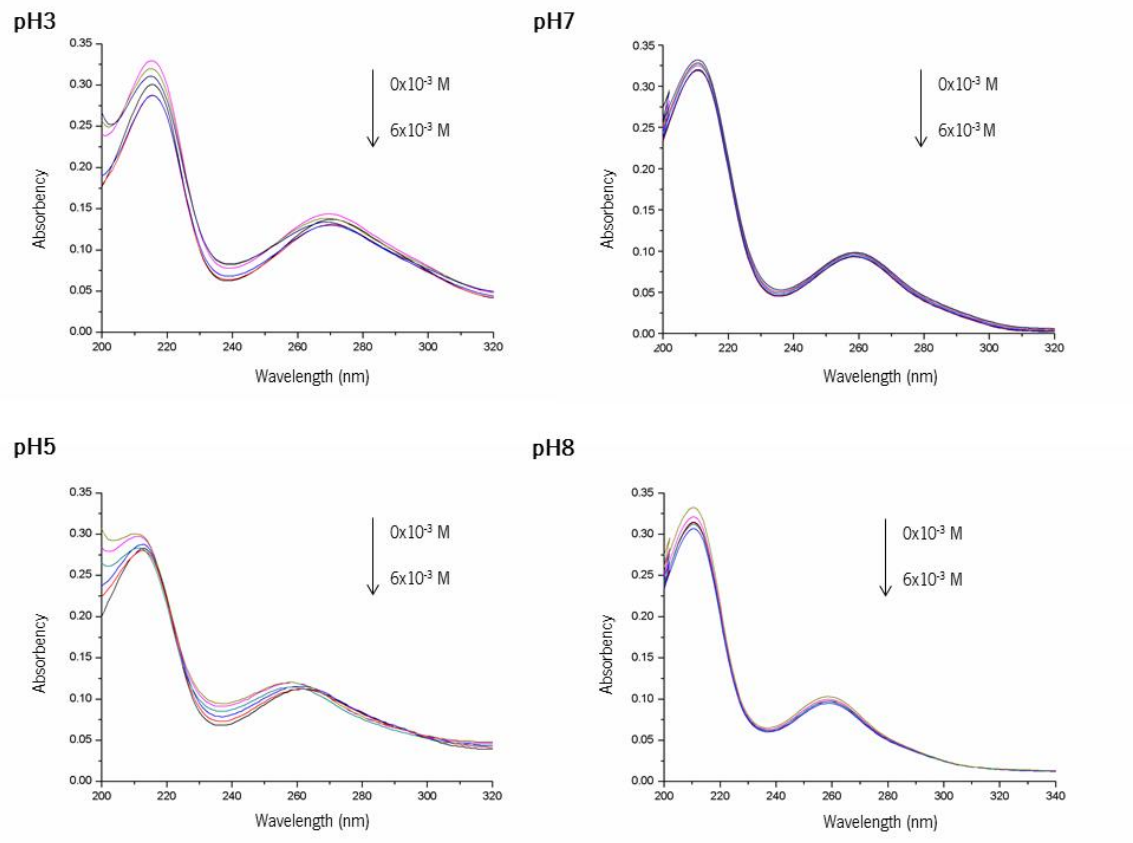

Fig 3.1.3 Gallic acid (1x10^{-5} M) UV-Vis absorbance spectra in different βCD concentrations (0-6 x10^{-3}M) at pH 3, 5, 7 and 8 (buffer H_3PO_4/NaOH).

As referred above, no significantly alterations were observed on UV-Vis gallic acid spectra at pH 7 and 8 (Fig 3.1.3), probably due to the absence of IC or their lower stability. Thus, the inclusion complex formation was only characterized at pH 3 and 5.

The UV-Vis absorbance spectra enable the confirmation of IC formation between of gallic acid and βCD. However, the biding strength and alterations caused by complexation on the guest properties lack further analyses. Thus, the stoichiometry, K and ΔG were used to estimate the stability of the IC [38]. The stoichiometry and K were calculated based on UV-Vis absorbance spectra data transformed by the Benesi-Hildebrand equation (equation 3.1.1) and ΔG was determined based on equation 3.1.3.

A linear relation (Fig 3.1.4) was obtained for the two pH values (3 and 5), indicating that 1 molecule of gallic acid complex with 1 molecule of βCD (IC 1:1).

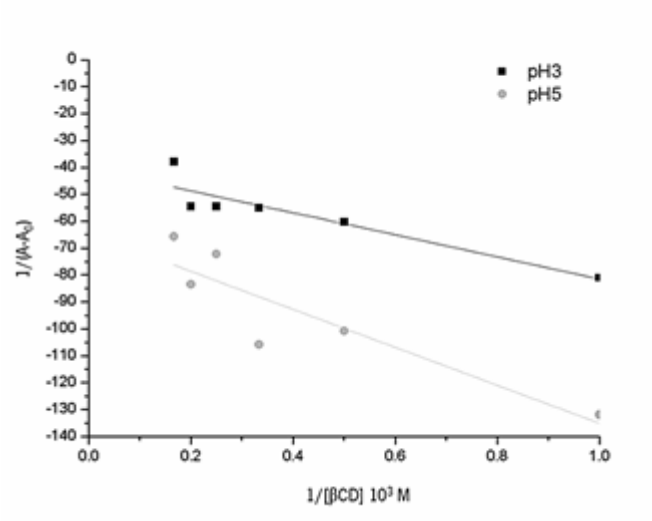

Fig 3.1.4 Benesi-Hildebrand plot for the gallic complexation with βCD, at pH 3 and 5 (buffer H_3PO_4/NaOH).

The pH range analysed (3 and 5) had lower influence on the K and ΔG, the values obtained were similar. The highest values were obtained for pH5 (K 71 M^{-1} and ΔG -10.4 kJ), however with r^2 0.865. At pH 3, the linear relationship obtained was better (r^2 0.957) but the K and ΔG were lower (K 41 M^{-1}, ΔG -9.83 kJ). The ICs formed at pH 3 or 5 were formed spontaneously by an exothermic reaction (ΔG<0) [39]. The ICs formation involves the replacement of polar water molecules, from the hydrophobic CD cavity, by gallic acid. At acids environments, gallic acid is present, mainly, has a neutral species (pK 3.4) [28], thus the encapsulation may be enhanced. Since, the K and ΔG for both pH were very similar, the pH 3 was considered the best condition for the βCD/gallic acid complexation due to it higher r^2.

From authors knowledge, there are just a few publications regarding gallic acid interaction with βCD [15, 18, 29]. Those works obtained similar parameters values for the IC characterization and, also, described analogues mechanisms of encapsulation. They all stated that the gallic acid was completely inside the βCD cavity, the carboxylic group towards to the small CD opening and 3 OH were placed near wider entrance (Fig 3.1.5). In the present work, the major interactions between the gallic acid and βCD were hydrophobic, since the βCD was not charged at the pH range describe. Therefore, the ICs stability were maintained by hydrophobic bonds between gallic acid and the cavity, as well as hydrogen bonds established between the gallic acid and the CD

OH groups [18, 29]. Thus, the gallic acid neutral species (pH 3) may be capable of deeper location on the CD cavity than it ionisable form (pH >3.4) (Fig 3.1.5). This fact justify the lower stability observed for the IC formed at pH5 and the lack of encapsulation at higher pH. Based on our results and on the referred works, the stability of IC βCD/gallic acid may be enhanced by acid environment (pH lower than 3.4).

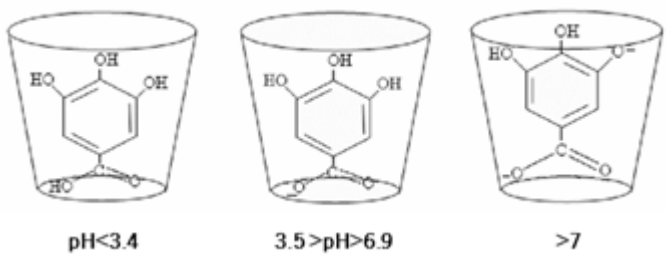

Fig 3.1.5 Schematic representation of gallic acid location inside βCD molecule at different pH.

Gallic acid Encapsulation by HPβCD and MβCD

The encapsulation of polyphenolics by modified cyclodextrins has been described as more stable than the ones obtained with native cyclodextrins [8, 40]. In the present work, 2 βCD derivatives (HPβCD or MβCD) were chosen based on their improved solubility and, also, because the substitutions enlarged the cavity opening, reducing the strong intramolecular hydrogen bond network. These characteristics facilitate the access of the guest molecule (in this case gallic acid) to the cavity, leading to the formation of a complex with higher stability. From author's knowledge, the inclusion of gallic acid by HPβCD or MβCD was analysed by the first time, on the present work.

The effect of pH (3, 5, 7 and 8) on the gallic acid interaction with HPβCD and MβCD was studied. The variations on phenolic UV-Vis spectra induced by those CDs at pH 5, 7 and 8 were not detected on the conditions tested (data not show). Additionally, the gallic acid encapsulation with the native CD (βCD) was more efficient at pH 3. Hence, this pH value was chosen to study the effect of the βCD modifications on the complexation with gallic acid (Fig 3.1.6).

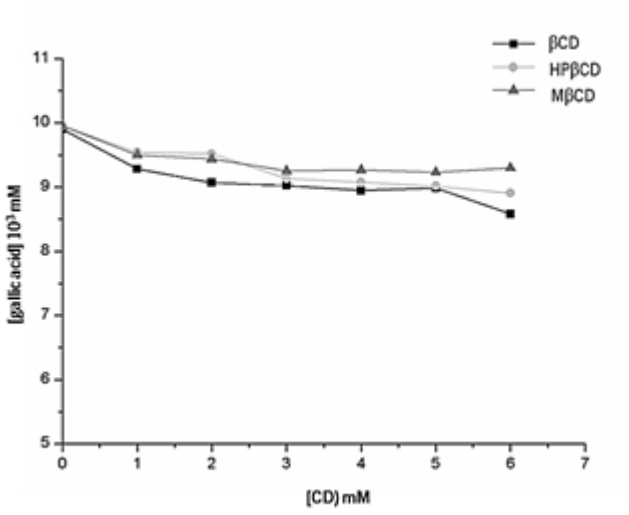

Fig 3.1.6 Gallic acid concentration detected on the solutions (based on the absorbency measured at the λ_{max}) after 24 h of complexation with different concentrations of cyclodextrins (0 to 6 mM) and at pH 3 (buffer H_3PO_4/NaOH).

The gallic acid behaviour was similar within the presence of the 3 CDs. A reduction of the gallic acid concentration on solution with the increased of CDs concentration was observed. These values were obtained from the UV-Vis spectra, where it was observed hypochromic effect (reduction of λ_{max} intensity) with the increase of CDs concentration. This effect may result from the interference with molecular groups, responsible for the UV-Vis absorbance, induced by encapsulation [19]. The same behaviour was reported above for the native CD.

Fig 3.1.6 suggests that the IC formed by the 3 CDs were similar, but additional characterization (stoichiometry, K and ΔG) of the complexes indicates differences among the CDs. The ICs obtained (HPβCD/gallic acid and MβCD/gallic acid) had a 1:1 stoichiometry, concordant results with the native CD. Though, the stability and thermodynamic parameters were different for these 2 CDs. The HPβCD K (90 M^{-1}) was higher than the βCD, and showed a lower ΔG (-11.0 kJ). These point out to the higher ability HPβCD to complex with gallic acid, when compared with the native CD. The size of HPβCD molecular cavity is similar to the βCD (7 glucopyranose units). Thus, the higher K obtained for the HPβCD suggest that the hydroxypropyl groups play a major rule on the IC process, these groups maintain the gallic acid molecules may be trapped inside the cavity [41]. The opposite was observed for the MβCD, since lower stability parameters were obtained (K 37 M^{-1} and ΔG -8.8 kJ). This CD was less favourable for the complexation of gallic acid, when compared with the native and the HPβCD.

Based on the results present above and as expected, the pH plays a major rule on the encapsulation of gallic acid by cyclodextrins. The pH 3 provide the best environment for the IC formation with the 3 CDs, and the pH 5 created conditions to the complexation between gallic acid and the native CD, with similar parameters achieved at pH 3.

Antibacterial Efficacy of CD/Gallic acid Complexes

The cyclodextrins encapsulation, usually, improve the guest molecule physicochemical and biological properties. Zhao [21] proved that the anti-oxidant and antibacterial activity of chlorogenic acid was not affect by its encapsulation by βCD. The antibacterial activity of the IC βCD/gallic acid and HPβCD/gallic acid was assessed by quantitative method. In order to ensure that gallic acid will be capable of killing all the bacteria cells on an infected wound, the environmental condition less favourable to its action (pH 3) was used for this analysis. Moreover, this pH allowed the best complexation process between gallic acid and βCD or HPβCD.

The IC complexes were prepared with equimolar solutions (1 mM, two folds the MBC pH 3) of CD and gallic acid, considering the stoichiometry determinate above (1:1). As expected, the controls (buffer, βCD and HPβCD) and no influence on the bacteria growth.

Fig 3.1.7 displays the susceptibility of the 3 bacteria when exposed to the ICs. Both ICs (βCD/gallic acid and HPβCD/gallic acid) were capable of reducing the 3 bacteria growth, but their antibacterial activity was constrained by the bacteria used. Against the gram negative bacteria (*K. pneumoniae*), the ICs had the same effect of the free gallic acid, completely growth inhibition. Regarding the gram positive bacteria (*S. epidermidis* and *S. aureus*), the antibacterial activity of gallic acid was reduce by βCD encapsulation, still the number of CFU detect was less than 3 log when compared with the control. The other IC (HPβCD/gallic acid) retained the gallic acid activity against *S.epidermis*, but allowed the growth of 1 log of *S. aureus*.

The gram positive and negative bacteria differ in their cell wall and, consequently, on their susceptibility to antimicrobial agents. The gram positive bacteria have a continuous cell wall of a thick layer of peptidoglycan, while gram negative bacteria have a non-continuous cell envelope formed by a thin layer of peptidoglycan covered by an outer membrane. Hence, it is expected that gram negative bacteria are more susceptible to antimicrobial agents than gram positive ones [42]. Therefore, the higher susceptibility of the gram negative bacterium (*K. pneumoniae*) to the

ICs activity suggest that the ICs were capable of reach the cell surface more efficiently and consequently, higher uptake, when compared to the other bacteria.

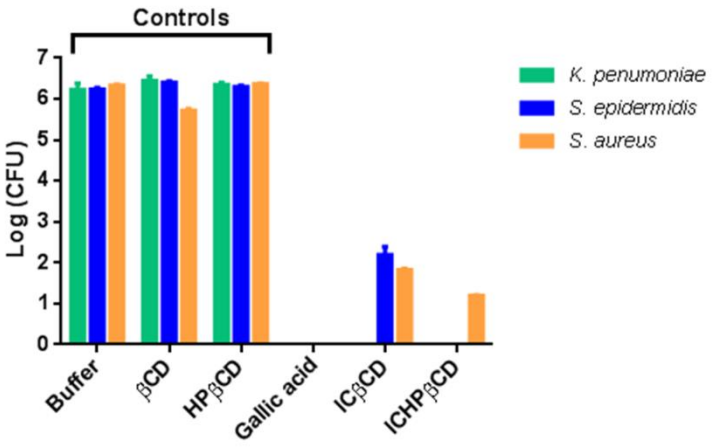

Fig 3.1.7 Quantitative analysis of the βCD/gallic acid and HPβCD/gallic acid inclusion complexes against *K. pneumonia*, *S. epidermidis* and *S. aureus* ($5x10^5$ cel.mL^{-1}). The inclusion complexes were prepared at pH 3 (buffer H_3PO_4/NaOH) with gallic acid and CDs at the same molecular proportion (1 mM). All data is expressed as mean + standard deviation (n = 9).

The reduction on the antibacterial activity of gallic acid regarding the ICs in contact with the gram positive bacteria may relay on the phenolic lower availability to interact with bacterial cells. As referred above, the antibacterial mechanism reported for gallic acid involves the interaction with cell surface, altering it electrochemical potential, reducing the membrane integrity, and hyperacidification of cytoplasm via proton donation that interfere with crucial metabolic pathways. Thus, assuming that gallic acid was complete inside the cyclodextrins and within a stable complex, as described above, the proton exchange was, probably, lower than free gallic acid. The HPβCD allowed a better interaction of gallic acid - bacteria, since the IC with this CD were capable of preserve gallic acid acidity against 2 bacteria, and the growth of *S. aureus* obtained was minimal.

Nevertheless, the encapsulation of gallic acid by the βCD and HPβCD may be viable option for the application as antibacterial agent.

Conclusion

The role of the solvent and pH on gallic acid properties was confirmed in this work. The intensity of phenolic acid peaks was modified by the buffer. The H_3PO_4/NaoH buffer allowed a better detection of the pH effect on the UV-Vis spectra.

The encapsulation of gallic acid by βCD was study at pH 3, 5, 7 and 8, and it was observed that the basic environment (pH 7 and 8) were less suitable for encapsulation. At pH 3 and pH 5, the ICs obtained had similar stability (K and ΔG values alike), though the linear relation was better at acid pH. Regarding the βCD derivatives, the encapsulation was, only, detected at pH 3. Therefore, the gallic acid neutral form appears to enhance its ability to form IC with HPβCD and MβCD. All the complexes obtained had 1:1 stoichiometry regardless the CD, but the IC stability parameters were higher for HPβCD and lower MβCD.

Moreover, the IC βCD/gallic acid and HPβCD/gallic acid antimicrobial activity was also analysed and the phenolic activity was retained by the ICs. Nevertheless, the HPβCD IC had better efficiency against the 3 bacteria and, also, had the better stability parameters.

Consequently, HPβCD/gallic acid may be a viable option for the improvement of gallic acid applicability as antibacterial agent for the treatment of skin and soft tissue infections.

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Chapter 3.2

Evaluation of antibacterial activity of caffeic acid encapsulated by β-Cyclodextrins

THE PRESENT CHAPTER WAS ADAPTED FROM THE FOLLOWING PAPER

Pinho E, Soares GMB, Henriques M. Evaluation of antibacterial activity of caffeic acid encapsulated by β-cyclodextrins.

International Journal of Biological Macromolecules (submitted)

Abstract

Caffeic acid has been described as antibacterial but this bioactive molecule has some issues regarding solubility and stability to environmental stress. Thus, encapsulation devices are required. This work goal was to study the effect of the caffeic acid encapsulation, by cyclodextrins, on the phenolic antibacterial activity.

The interactions between the caffeic acid and 3 cyclodextrins (βCD, HPβCD and MβCD) were study. The pH effect on the inclusion mechanism was, also, analysed. The formation of an aqueous soluble inclusion complex was confirmed for both βCD and HPβCD with a 1:1 stoichiometry. The βCD/caffeic acid complex showed higher stability than HPβCD. Regarding the environmental conditions; pH 5 was the most suitable for the inclusion complex formation. The antibacterial activity of caffeic acid was similar at pH 3 and pH 5 against 3 bacteria (*K. pneumoniae, S. epidermidis and S. aureus*), being *S. epidermidis* the most susceptible bacterium to this phenol. The antibacterial activity of the inclusion complexes was described here for the first time, and it was shown that the caffeic acid activity was remarkably enhanced by the cyclodextrins encapsulation.

Keywords: Antibacterial activity, Benesi-Hildebrand equation, caffeic acid, cyclodextrin, inclusion complex.

Introduction

Wounds allow the microorganism deposition and growth, causing skin and soft tissues infections and, consequently, a delay on the healing process. Over the years, antibiotics have been indiscriminately used for the treatment those infections, and aggravated by extended therapies, leading to the development of microbial resistance [1]. Thus, antibiotics had been losing their capacity against pathogens, and new therapies against multi-resistant bacteria are hard to find. Therefore, natural antibacterial agents have become an alternative to overcome this issue [2].

Caffeic acid (3,4-dihydroxycinnamic acid) is a simple phenolic acid derived from the hydroxycinnamic acid, with some interesting biological properties, such as anti-oxidant, antibacterial and fungicide, being the latter the most studied [3–7].The biological activities of this phenolic acid have been related to its electrical charge. In solution, the caffeic acid, as well as the other phenolic compounds, lies in equilibrium between the protonated and deprotonated form, according to the environmental pH. At lower pH (2-3.5) the molecule is neutral and at higher pH the caffeic acid assumes a charge form (Fig 3.2.1) [7]. Some authors [8, 9] reported that the antibacterial activity of this phenolic increase as the pH decrease (pH range: 4.5 -7). This biological active of caffeic acid can be compromised by the sensibility to oxidation and acidic environments, as well by its poor solubility in water [10].

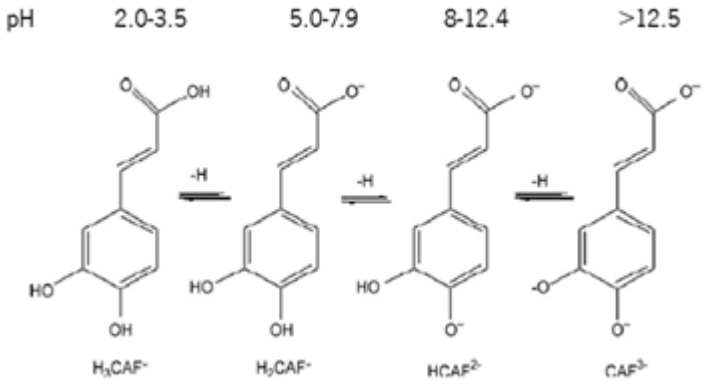

Fig 3.2.1 Caffeic acid equilibrium in aqueous solution.

The improvement of physical, chemical and biological properties of natural bioactive molecules can be accomplished by their encapsulation within cyclodextrins (CD). Moreover, CDs has been

described as safe to humans and approved by FDA (Food and Drug Administration) [11].These cyclic oligosaccharides present a hydrophilic external surface and hydrophobic cavity, which make them capable of complex with both hydrophilic and hydrophobic molecules. CDs interact with the bioactive molecules forming an inclusion complex (IC), the complex's stability results from the perfect physical fit between the CDs cavity and the molecule and, also, from the interaction established between the 2 molecules [12]. All these factors contribute to a favourable thermodynamic system, which can be characterized by the enthalpy (ΔH), entropy (ΔS) and Gibbs free energy (ΔG) [13]. Moreover, the pH and the solvents are, also, crucial for the efficiency of the complexation process between organic compounds and CDs [11, 14, 15]. In nature, it is possible to find 3 native CDs (α, β and γ), but a wide range of modifications have been carried out on native CDs to improve their properties. βCD has been the most applied in industry, as well as its derivatives [11]. In the present work, two derivatives were chosen, hydroxypropyl-β-cyclodextrin (HPβCD) and methyl-β-cyclodextrin (MβCD), since both CDs has higher solubility than the βCD (500 and 750 $g.L^{-1}$ at room temperature compared to 18 $g.L^{-1}$ for βCD), which enhance the complexation with poor water soluble molecules [16].

Therefore, the present work aims to study the effect of the pH on the formation of the IC between the caffeic acid and 3 CDs (βCD, HPβCD and MβCD), characterized by UV-Vis spectrometry. Moreover, the effect of the encapsulation of caffeic acid by the cyclodextrin on its antibacterial activity was, also, studied.

Material and methods

Material

Caffeic acid (3,4-dihydroxycinnamic acid) was purchased from Sigma, β-cyclodextrin (βCD, 1135 $g.mol^{-1}$) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, 1309 $g.mol^{-1}$) were acquired from AppliChem, and the methyl-β-cyclodextrin (MβCD, 1310 $g.mol^{-1}$) was obtained from Wacker. Caffeic acid stock solution ($1x10^{-3}$ M or $2.3x10^{-1}$ M) was prepared in ethanol and maintained 30 min at 50 ºC and 200 rpm. Stock solutions of each CD ($4x10^{-2}$M) were prepared in distilled water, βCD solution was maintained at 50 ºC and 200 rpm, during 30 min in order to improve it solubility in water. Buffers were prepared by mixing proper amounts of both H_3PO_4 ($1x10^{-2}$ M, pH 2.05) and NaOH (1 M, pH 14), until the desired pH (3 and 5).

INCLUSION COMPLEX PREPARATION

In order to determine the stoichiometry and stability constants of the 3 ICs, solutions with different concentrations of each CD (between 0 and $6x10^{-3}$ M) were added to the same concentration of caffeic acid ($1x10^{-5}$ M), at each pH (buffer H_3PO_4/NaOH). The solutions were placed in ultrasounds bath during 30 min, and, after, they were maintained 24 h at 25 ºC and 50 rpm, on dark. Samples of each solution were taken (0 and 24 h) for absorbance measurements.

The absorption spectra of each solution were recorded on a Jasco V560 spectrometer in the range 200–360 nm, using a 1cm quartz cuvette.

ANTIBACTERIAL ACTIVITY ASSESSMENT

Bacterial Suspension

The antibacterial activity of caffeic acid on buffer H_3PO_4/NaOH at each pH (3 and 5), as well as the ICs (prepared according with the conditions described above and with equimolar concentrations, $2.3x10^{-3}$ M, of caffeic acid and CDs, at pH5) was tested against 3 bacteria: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296). The bacteria were grown in tryptic Soy Agar (TSA, Merck, Germany)

for 24 h at 37 °C. The cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 18 h at 37 °C under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to $1x10^{6}$ cells.mL^{-1}, via absorbance readings, using the correspondent calibration curve.

Susceptibility of Caffeic Acid

The MBC was obtained according to the method described by Wiegand et al. [17], an adaptation of the standard methods published by Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST, 2000), using the broth microdilution procedure. Thus, working solutions of 0.28 M of caffeic acid were prepared at pH 3 and pH 5 (buffer H_3PO_4/NaOH). Serial dilutions of these solutions were made with Mueller–Hinton broth (MHB, Merck, Germany) to a final volume of 50 µL. Afterwards, 50 µL of each bacterium suspension were added to a final concentration of $5x10^{5}$ cell.mL^{-1}. Caffeic acid- and bacteria-free controls were also included. The plates were incubated for 24 h at 37 °C. The number of viable cells, was assessed by determination of the number of CFUs, by plating 10 µL of cell suspension from each well onto TSA, and incubated for 24 h at 37 °C.

The procedure was made in triplicate for each pH and bacteria combination in, at least, 3 independent assays.

Antibacterial Activity of Inclusion Complexes

The antibacterial activity of the ICs against the 3 bacteria was firstly assessed by the Disc diffusion method described by the National Committee for Clinical Laboratory Standards (NCCLS), M2-A8 document [18], with some modifications. The TSA was the nutritive media used, and 200 µL of each inoculum ($1x10^{6}$ cells.mL^{-1}) was spread on these media plates. Sterile filter paper disks (“Blanck Discs”, Liofilchem, Roseto, Italy, 6 mm in diameter) were placed over the petri dish, and impregnated with 20 µL of each IC. The plates were incubated at 37 °C for 24 h. Thereafter, the size of the inhibition halo was measured.

The ICs capacity to destroy the bacteria was also measured quantitatively. A volume of 50 µL of each complex (IC βCD/caffeic acid and IC HPβCD/caffeic acid) was added to 50 µL of $1x10^{-6}$ cells.mL^{-1} of each bacterium, on 96 well plate. Bacteria and medium controls were also included.

The plates were incubated for 24 h at 37 ºC. The antimicrobial activity of each solution was assessed by determination of the number of CFUs, as described above. The procedure was made in triplicate for each bacterium at least in 3 independent assays. Solutions of buffer at pH 5, caffeic acid ($2.3x10^{-2}$ M), βCD ($2.3x10^{-2}$ M) and HPβCD ($2.3x10^{-2}$ M) were also included, to ensure that none of these factors, alone, could have influence on the antibacterial activity of the ICs.

Results and Discussion

CAFFEIC ACID

pH Effect on the UV-Vis Absorption Spectrum

The UV-Vis spectroscopy analyses the alterations of the electronic energy levels within the molecules, as a result of the electrons transference to different orbitals [19]. In the case of polyphenols, their spectra result from electronic transitions between π-type molecular orbitals [20]. The caffeic acid spectra, usually, shows 3 peaks: one at 217nm which is associated with the π-π transition of the phenyl group; the peak near 290 nm attributed to the π-π transition of the phenolic group; and the last one (320 nm) is, normally, associated to the double-bond π-π transition [21].

Since the pH can change the UV-Vis spectra of polyphenols [22], the caffeic acid spectra were recorded between 200 and 360nm for solutions of pH 3 and 5 (Fig 3.2.2). Regarding the peaks associated to the π-π transition of the double bound (near 320 nm), and the phenolic group (near 288 nm), the pH increase induced a reduction of their intensity and had a hypsochromic effect (shift of the peak towards shorter wavelength). A bathochromic effect (shift of the peak towards longer wavelength), and increased intensity was observed for the peak attributed to the π-π transition of the phenyl group, when the pH was increased.

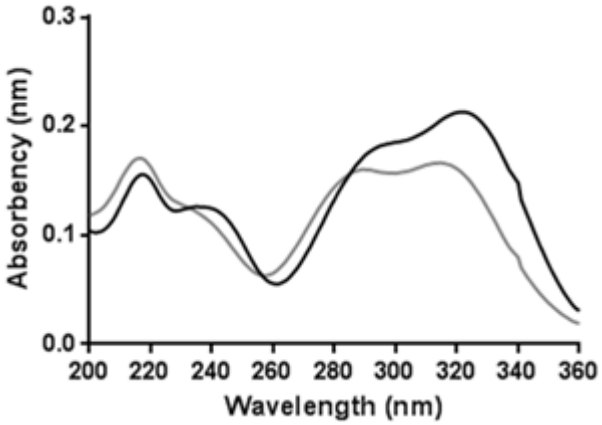

Fig 3.2.2 Absorbency spectrum of caffeic acid (1x10^{-5} M) dissolve in H_3PO_4/NaOH buffers at pH 3 (black) and pH 5 (grey).

The alterations caused by the pH on the caffeic acid UV-Vis spectra have been attributed to the two adjacent hydroxyl groups (OH) linked to the benzene ring (Fig 3.2.1). Those changes may result from the chemical alteration of the molecule, for instance the blue shift of the 220 nm peak with pH 5 may be a consequence of the formation of unstable quinone intermediates or other resonance forms [19, 22].

Antibacterial Activity: pH Influence

The biological properties of the phenolic compounds strongly depend on the environmental pH [22]. Therefore, the effect of pH on the antibacterial activity of caffeic acid against 3 of the most common bacteria isolated from wound infections, was assessed. The range of pH tested had no visible influence on the caffeic acid activity. The MBC was the same for pH 3 and 5 for the 3 bacteria. Being the *S. epidermidis* the most susceptible bacteria to caffeic acid (0.018 M) and the other 2 bacteria had the same behaviour when exposed this phenol, 0.07 M was need to destroys all their cells.

The antibacterial ability of caffeic acid has been related to its capacity to cause hyperacidification of the environment. The acidification of the plasma membrane, via proton donation, result on disruption of the bacteria cell and the same effect on the intracellular cytosolic, leads to inhibition of the enzyme H^+-ATPase necessary for the ATP production [23–25]. Also, caffeic acid can change the electric potential of the bacterial membrane by quench free electrons from the electron transport chain or by interfering with the proton efflux by dehydrogenases inactivation. Because of this, the bacteria growth may be reduced or even inhibit, since the oxidative phosphorylation reaction cannot occurs [23, 26].

According to our results, the antibacterial activity of caffeic acid may be related to the phenolic part of the molecule, since the changes observed on the molecule are on the carboxylic group, at the pH tested (Fig 3.2.1). Furthermore, this part of caffeic acid (carboxylic group) has the ability to donate the protons associated with the acidification of the environment.

CAFFEIC ACID INCLUSION COMPLEXES

Effect of Cyclodextrins and pH on the UV-Vis Absorption

Given the differences obtained above, both pH values were used for the study of the IC formation between the phenolic compound and the 3 CDs. The encapsulation process involving CDs induces alterations on physical-chemical properties of the guest molecule (caffeic acid). In the case of UV-Vis spectrometry, the absorbance intensity of the guest molecule is changed by its presence within the CD cavity [27].

Caffeic acid absorbency was not altered by the increase in MβCD concentration (data not shown), so the encapsulation of caffeic acid with this CD was not detectable, at the conditions tested. Thus, MβCD was not included on the further analysis. Fig 3.2.3 displays the UV-Vis absorption spectra of caffeic acid, in the present of different concentrations of βCD and HPβCD. Since the changes on the spectra were more consistent at the peak near 220 nm, this peak was used for the absorbance analysis.

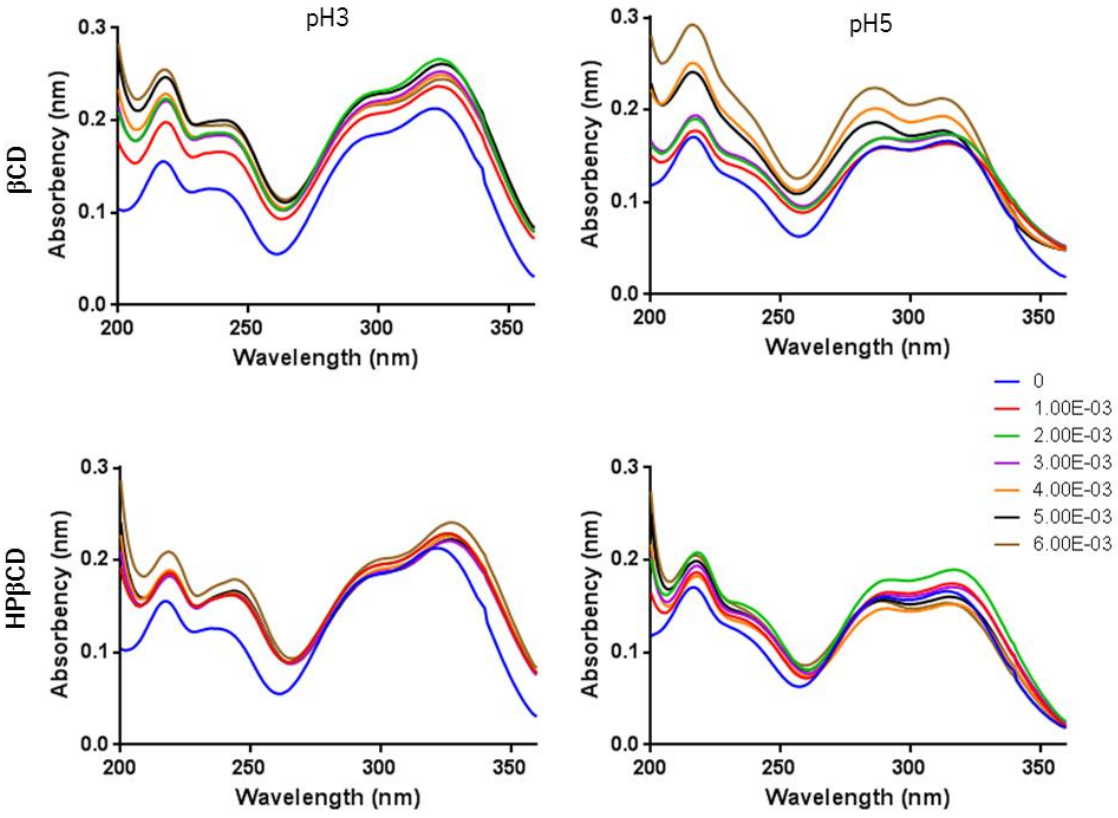

Fig 3.2.3 Absorbance spectra of caffeic acid (1x10^{-5} M) in different cyclodextrin concentrations (0-6 x10^{-3} M) at pH 3 and 5.

Regardless the pH or the type of CD, as CDs concentration increased the intensity of the peak, also, increased, and a slightly red-shifted (peak shift to a longer wavelength) was observed. The last alteration was more notorious at pH 5. Those changes on caffeic acid spectrum may result from the partial protection of the molecule by the CD cavity, as well as the increase of the phenolic compound solubility, factors that suggest the IC formation between this phenolic and both CDs [28].

Inclusion Complexes Characterization

The complexes were assessed based on the phase-solubility method described by Higuchi and Connors [29]. This technique analyses the alterations of the caffeic acid (guest) properties (by of UV-Vis absorbency spectrometry), in mixtures with excess of the guest and different concentrations of the host (CDs). The ICs were characterized based on the stoichiometry, K (stability constant) and ΔG (Table 3.2.1). The first 2 parameters were calculated using the modified Benesi-Hildebrand equation [30] (equations 3.2.1 and 3.2.2):

(3.2.1) $$\frac{1}{A-A_0} = \frac{1}{A'-A_0} + \frac{1}{K(A'-A_0)[CD]_0}$$

(3.2.2) $$\frac{1}{A-A_0} = \frac{1}{A'-A_0} + \frac{1}{K(A'-A_0)[CD]_0^2}$$

[CD]₀ Cyclodextrin initial concentration

A Absorbance intensities in the presence of cyclodextrin

A₀ Absorbance intensities without cyclodextrin

A' Limiting intensity of absorption

Base on the equation (3.2.1), a double reciprocal Benesi-Heldebrand plot, i.e. $\left(\frac{1}{A-A_0}\ vs\ \frac{1}{[CD]}\right)$ were draw (Fig 3.2.4). Since the better fit was obtained for this plot, the graphics of $\left(\frac{1}{A-A_0}\ vs\ \frac{1}{[CD]^2}\right)$ were not showed.

Fig 3.4 shows a straight line with good correlation, indicative of 1:1 molecular complex between caffeic acid and both CDs. The K (Table 3.2.1) was obtained based on the slope of the line and can be used as a measurement of the stability of the complexes [31]. Thus, based on our results,

βCD appears to be a most suitable for the complexation of caffeic acid. Moreover, the pH had lower influence on the IC formation with these CDs, but the K value was slight superior at pH 5.

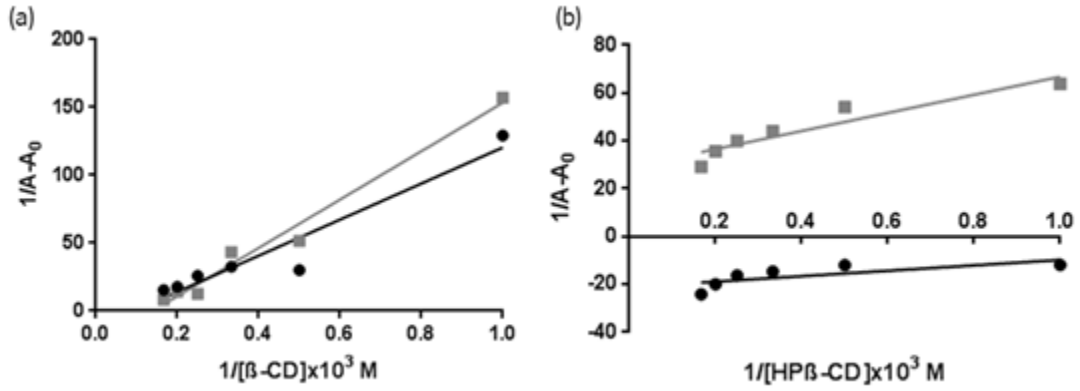

Fig 3.2.4 Benesi-Hildebrand plot for the caffeic acid complexation with βCD (a) and HPβCD (b), at pH 3 (black) and pH5 (grey).

The Gibbs free energy was obtained from the equation (3.2.3). In concordance with K values, the pH had lower influence on the thermodynamic parameter assessed (Table 3.2.1). The pH effect was more obvious for the HPβCD, where the IC was enhanced by pH 5.

(3.2.3) $\Delta G = -RTln(K)$

ΔG Gibbs free energy

R gas constant

T temperature (Kelvin)

K stability constant

The mechanism responsible for the IC formation between CDs and the guest molecule (in our case the caffeic acid) involves the substitution of enthalpy- rich water molecules from the central cavity by caffeic acid. An exothermic reaction occurs as a result of energy release by the system. Thus, the IC were stabilized mainly by van der Waals interactions and hydrogen bonds [11].

The native CD (βCD), as well as the HPβCD, at the pH range used are not charged, so hydrophobic interactions between the CD cavity and the caffeic acid and hydrogen bonds of the caffeic with the OH of the CDs were the mainly forces responsible by the complex stabilization.

According to our results and other published works [7, 27, 32], the caffeic acid aromatic part, including the double bond, was inside of both CDs cavities and the more polar group (carboxylic group) was projected to the water phase.

Table 3.2.1. Parameters for the inclusion complexes characterization. r^2: coefficient of determination, K: stability constant and ΔG: Gibbs free energy

	βCD		HPβCD	
	pH 3	pH 5	pH 3	pH 5
r^2	0.923	0.979	0.548	0.924
Stoichiometry	1:1	1:1	1:1	1:1
K (M^{-1})	133	178	10	37
ΔG (kJ)	-12	-13	-6	-9

At pH 3, the neutral form of the caffeic acid (Fig 3.2.1) is predominant and at pH 5 caffeic acid assumes a monoanionic form, thus the hydrophobicity of the phenolic molecule reduces with the increase of pH [7]. Therefore, pH 5 was more suitable for the formation of a IC more stable, as also reported by Górnas et al [27].

Based on the above results, the βCD appears to be the most suitable CD for the encapsulation of caffeic acid. Since, the changes induced on the UV-Vis spectra of caffeic acid by the concentration of this CD were more notorious than the ones observed for HPβCD. Additionally, the K and the ΔG were, also, higher for the βCD/caffeic acid complexes. The higher efficiency of complexation by the βCD may be a result of the better fit of caffeic acid molecule into βCD cavity, without the interference of the HPβCD hydroxypropyl groups.

Inclusion Complexes Antibacterial Activity

From author's knowledge, the effect of CDs on the antibacterial activity of caffeic acid was described here for the first time. Since the pH had no significant influence on the caffeic acid activity and the pH 5 allowed a formation of a more stable IC for both CDs (Fig 3.2.4 and Table 3.2.1), this pH was used for the assessment of the antibacterial activity of the IC (βCD/caffeic acid and HPβCD/caffeic acid).

As reported above, the IC formed between caffeic acid and βCD or HPβCD were 1:1, thus, solutions with the $2.3x10^{-2}$ M of each intervener on the reaction was used. The concentration of

caffeic acid used was lower than the MBC, reported above, for *K. pneumoniae* and *S. aureus., s*ince βCD has a solubility limit in water of $2x10^{-2}$ M at room temperature [16].

The qualitative assessment of the antibacterial activity of the complexes (Fig 3.2.5) exhibited a presence of growth inhibition halo for all the IC, higher than the halo obtained for the caffeic acid alone. The only exception was the IC HPβCD/caffeic acid when in contact with *S. aureus*, in this case no halo was observed. This type of method has some drawbacks regarding the diffusion of the antibacterial agents [33], in this case the complexes. Therefore a complementary quantitative analysis was made.

Fig 3.2.6 display the antibacterial activity of the two ICs (βCD/caffeic acid and HPβCD/caffeic acid) as well as the controls (buffer, caffeic acid, βCD and HPβCD). As expected the buffer and the CDs alone had no antibacterial activity. The free caffeic acid was only capable of killing all the *S. epidermidis* and induced a slight reduction on the growth of the other two bacteria, since the concentration used was six times less ($1.15x10^{-2}$ M) than MBC ($7x10^{-2}$ M) for *K. pneumoniae* and *S. aureus*, as explained above.

Interestingly, the IC of the caffeic acid with both CDs were capable of destroy all the cells from the 3 bacteria (Fig 3.2.6), even if we used less than half of the MBC concentration for the *S. aureu* and *K. pneumoniae* on the IC preparation. The encapsulation of caffeic acid by the CDs enhanced it antibacterial activity against *S. aureus* and *K. pneumoniae*.

The antibacterial activity of caffeic acid appears to be deeply linked with the molecule ability to interact with electrons presents on the bacteria surface or inside the cytoplasm [34]. The enhanced antibacterial activity of the βCD/caffeic acid and HPβCD/caffeic acid may be a result of the improved solubility of the caffeic acid and reduction aggregates formation and, also, by the higher availability of the caffeic acid to interact with bacteria, all induced by the CDs encapsulation.

Therefore, the encapsulation of caffeic acid by the βCD and HPβCD appears to be viable option for the improvement of this phenolic antibacterial activity, and further application as antibacterial agent.

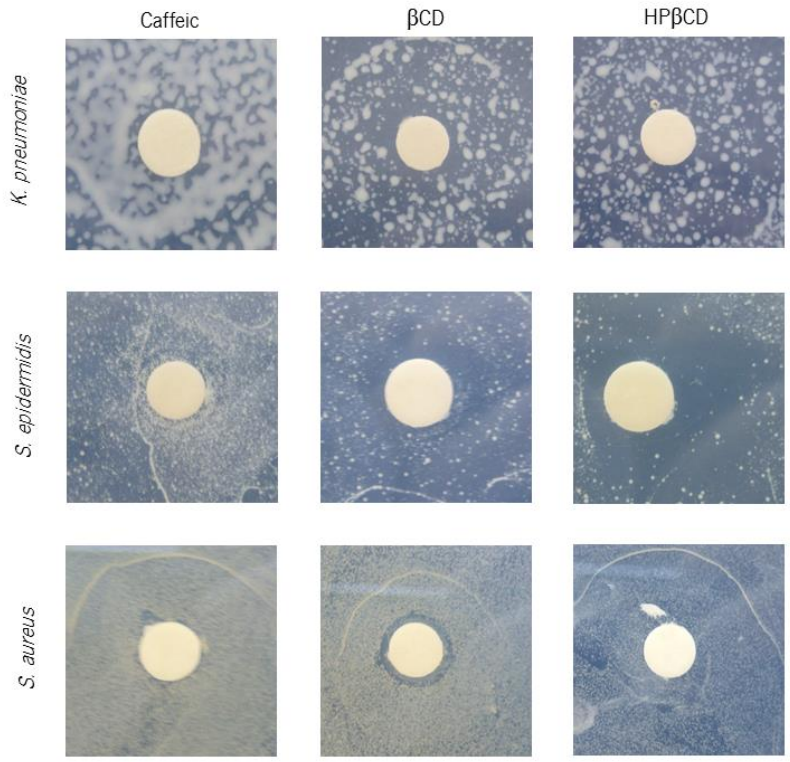

Fig 3.2.5 Disk diffusion assay of the βCD/caffeic acid and HPβCD/caffeic acid inclusion complexes against *K. pneumonia*, *S. epidermidis* and *S. aureus* ($1x10^{6}$ cel.mL^{-1}). The inclusion complexes were prepared at pH 5 (buffer H_3PO_4/NaOH) with caffeic acid and CDs at the same molecular proportion ($2.3x10^{-2}$ M).

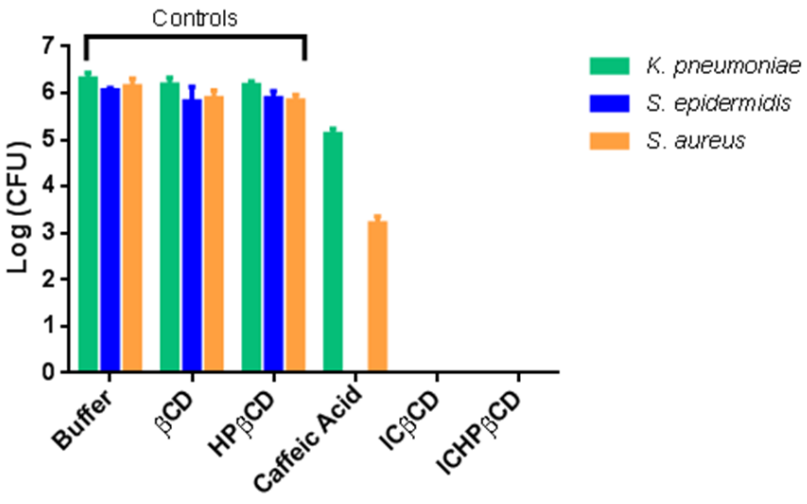

Fig 3.2.6 Quantitative analysis of the antibacterial activity of the complexes βCD/caffeic acid and HPβCD/caffeic acid against *K. pneumonia*, *S. epidermidis* and *S. aureus* ($5x10^{5}$cel.mL^{-1}). The inclusion complexes were prepared at pH 5 (buffer H_3PO_4/NaOH) with caffeic acid and CDs at the same molecular proportion ($2.3x10^{-2}$ M). All data is expressed as mean + standard deviation (n = 9).

Conclusion

In the present work, the IC between caffeic acid and the native CD (βCD) and 2 derivatives (HPβCD and MβCD) was characterized, based on the changes on UV-Vis absorption spectra of the phenolic compound. The results obtained showed that the MβCD was not capable of complex with caffeic acid, at the conditions tested. Moreover, the pH (3 and 5) showed lower influence on the complexation process with βCD and HPβCD, being the higher pH the most suitable. The complexes obtained had 1:1 stoichiometry regardless the CDs, but βCD complexes had higher stability parameters.

The caffeic acid was more effective against *S. epidermidis* than *K. pneumoniae* or *S. aureus* and the pH had no influence on its antibacterial capacity. Therefore, the carboxylic group may not be involved in the interaction caffeic acid – bacteria. Moreover, the antibacterial activity of the caffeic acid was enhanced by the complexation with βCD and HPβCD.

Although the encapsulation of caffeic acid by both βCD and HPβCD enhanced its antimicrobial activity, the IC βCD/caffeic acid appears to be the best option to increase the applicability of this phenolic as antibacterial agent against skin infections, due to its greater stability.

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Chapter 4

CD-based Hydrogels Load with Phenolic Acid for Wound Dressing Application

Chapter 4.1

Cyclodextrin/cellulose hydrogel with gallic acid to prevent wound infection

THE PRESENT CHAPTER WAS ADAPTED FROM THE FOLLOWING PAPER

Pinho E, Soares GMB, Henriques M. Cyclodextrin/cellulose hydrogel with gallic acid to prevent wound infection

Cellulose (Submitted).

Abstract

Cyclodextrin-based hydrogels have been described as suitable for the controlled-release of bioactive molecules for wound dressing proposes. These materials have major advantages, since they present hydrogels properties (high degree of swelling and easy manipulation) in combination with the encapsulation ability of cyclodextrins.

β-cyclodextrin (β) or hydroxypropyl-β-cyclodextrin (HPβ) were cross-linked (1,4-butanediol diglycidyl ether) with hydroxypropyl methylcellulose under mild conditions. The hydrogels were chemical characterized by swelling degree, FTIR, DSC and contact angle. The gallic acid loading and release was also analysed, as well the antibacterial and cytotoxicity of the polymeric networks.

The hydrogels obtained were firm and transparent, with good swelling ability. The gel-HPβ had a surface more hydrophilic when compared with the gel-β. Nevertheless, both hydrogels were capable to incorporate gallic acid and sustain the release for 48 h. The antibacterial activity of gallic acid was maintained after it adsorption within the polymeric matrix as well as it effect on fibroblast proliferation.

Therefore, gel-β and gel-HPβ conjugated with gallic acid were shown to be a viable option for antibacterial wound dressing.

Keywords: Antibacterial activity, 1, 4-butanediol diglycidyl ether, cyclodextrin, gallic acid, hydrogel, wound dressing.

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Introduction

Wounds provide suitable environment for the deposition and proliferation of pathogenic microorganisms, causing skin and soft tissues infections and triggering the patient immunological response [1]. The infections severity may range from self-limit superficial to life-threatening diseases. Moreover, skin and soft tissue infections increase the production of wound exudate and tissue deterioration, thus higher wound dressing replacement is required, causing pain to the patient and amplifying the probability of removing the newly formed skin [2]. Therefore, the research on wound dressings field has been focused on newly wound dressings capable of mechanical protection and suitable environmental conditions for proper healing and, additionally, capable of sustained delivery of antimicrobial agents to prevent wound infections [3].

Hydrogels have been, successfully, applied as wound dressings and drug delivery devices. They are polymeric networks with hydrophilic character, capable of absorb large amounts of water and with suitable physicochemical properties for contact with human tissue without causing injury [3]. However, hydrogels have some drawbacks as drug delivery systems, their capacity to load hydrophobic drugs is quiet reduced, as well as, the control over the drug release mechanisms (the diffusion is normally rapid and non-linear) [4]. So, cyclodextrins (CD) -based hydrogels have been synthetized to improve the drug delivery system. These materials benefit from the suitable swelling ability of hydrogels and from the encapsulation capacity of CDs [5]. CDs are truncated oligosaccharides with the ability to form inclusion complexes (IC) with a wide range of molecules, due to their hydrophilic surface and hydrophobic cavity. When use as monomer for hydrogel production, CDs can act, simultaneous, as carriers and as enhancers for the hydrogel stability [3].

Nevertheless, the methods used for the preparation of CD-based hydrogels, usually, involve high temperatures, leading to toxic products from undesirable side reactions, decreasing their applicability has biomedical devices [6]. Rodriguez-Tenreiro et al [5] developed a method for CD-based hydrogel synthesis with only 1 step, using condensation with ethylene glycol diglycidyl ether (EGDE) to obtained CD networks, under mild environmental conditions and without previous modification on the CD structure. CD-based hydrogels synthesized by this method showed good swelling and mechanical properties, and enhanced ability to load and release bioactive molecules [5–9].

Polyphenolics has been proposed has viable alternatives to broad-spectrum antibiotics normally used on wound infections treatment [10, 11]. Previous work, Chapter 2 and 3.1, showed that gallic acid (a simple phenolic acid) has good applicability as antibacterial agent against bacteria commonly isolated from wound infections. Although, its applicability on the pharmaceutical field has been constrained by gallic acid susceptibility to environmental factors and low solubility [12–14].

Therefore, the present work aimed to develop a material capable of forming inclusion complexes with gallic acid and kept the molecule's antibacterial properties, which could able to prevent wound infections and enhance the healing process.

Material and methods

Material

Gallic acid (3,4,5-trihydroxybenzoic acid) was provide by Merck, β-cyclodextrin (βCD, 1135 g.mol^{-1}) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, 1309 g.mol^{-1}) were acquired from AppliChem. Hydroxypropyl methylcellulose (HPMC) Methocel® K4M was purchased from VWR Prolab; 1,4-butanediol diglycidyl ether (BDGE), 50-60% in water was from Acros Organics. Purified water, obtained by reverse osmosis (MiliQ®, Mikipore, Madrid, Spain) with a resistivity above 18.2 MΩ cm^{-1}, was used.

Hydrogel Synthesis

The hydrogels were prepared based on the method described by Garcia-Fernandez [9]. Solutions of each CD, 2.5 g in 10 mL of NaOH (0.2 M), were prepared and maintained for 5 min, at 25 ºC and 200 rpm (mechanical agitation). The HPMC (0.025 g) was added to each solution and the solubilisation was improved by mechanical agitation (200 rpm) during 5 min at 25 ºC. The solutions (5 ml of each) were transferred to petri dishes and 2 mL of BDGE was added, to each one. The petri dishes were sealed with parafilm and maintained for 2 min at 25 ºC and under mechanical agitation (200 rpm). To complete the crosslink process, the plates were kept at 50 ºC for 12 h. At this temperature, the CD and HPMC stability was assured. After cooling, hydrogels were immersed in ultrapure water for 12 h and 25 ºC, to allow the swelling. Then, they were transferred to a HCl solution (10 mM) for more 12 h, and finally immersed in water for 7 days. The dry process was performed as followed: the hydrogels were kept at 25 ºC for 24 h and, after, were transferred to a desiccator until weight stabilization.

Swelling Determination

The amount of water absorbed by the hydrogels was calculated based on the difference between the weight of fully swollen hydrogel in water (W) and the weight after the dry process (W_0). For that, dry hydrogels (30 ± 3 mg) were weighted and immersed in 5 mL of ultrapure water. Their weight was recorded in regular time periods, until the weight stabilizes (fully swollen hydrogel),

using a Saitouris BL 1205 (d=0.1mg) scale, and after removing the water from the hydrogel surface.

The degree of swelling (Q) was calculated based on equation 4.1.1.

(4.1.1) $$Q = \frac{W - W_0}{W_0} x100$$

The swelling profile was obtained from the data recorded previously and plotting the amount of water absorbed at time t (W_t)/ amount of water at equilibrium ($W\infty$) versus time (min).

Gallic acid Loading and Release

Gallic acid solution ($2.3x10^{-2}$ M, in 2% methanol) was dissolved in H_3PO_4/NaOH buffer (pH 3 ± 0.5), and kept for 30 min in an ultrasonic bath, to allow the fully solubilisation of gallic acid. Dry hydrogel samples (30 ± 3 mg) were immersed in 5 mL of gallic acid solution, 25 ºC and 60 rpm. The amount of phenolic acid in the solution was assessed by UV-Vis spectrophotometry, until the absorbance values stabilize. The gallic acid loading (%) was calculated based on the variation of gallic acid on the initial solution and on the equilibrium.

The gallic acid release, from hydrogels, was performed using load dry samples. The samples (30 ± 3 mg) were immersed in 5 mL of synthetic sweat solution (SSS, 0.5 g /histidine monohydrochloride monohydrate, 5 g of sodium chloride, 2.2 g of sodium dihydrogen orthophosphate dihydrate, pH 5 ± 0.5). The samples were maintained at 25 ºC and the absorbance of the release medium was monitored until stabilization.

The absorption spectra were measured in the range of 200–360 nm and recorded on a Jasco V560 spectrometer, using a 1cm quartz cuvette.

HYDROGELS PHYSICOCHEMICAL CHARACTERIZATION

Fourier Transform Infrared Spectroscopy (FTIR)

Samples (0.5 ± 0.05 g) of each hydrogel, βCD-co-HPMC (gel-β) and HPβCD-co-HPMC (gel-HPβ) with or without gallic acid, were used for FTIR-ATR analysis. The spectra were recorded between 400 and 4000 cm^{-1}, in Avatar 360 FTIR spectrometer. The spectra of compounds alone (gallic

acid, βCD, HPβCD and HPMC) were, also, recorded, using the potassium bromide pellet technique.

Thermal Analysis by Differential Scanning Calorimetry (DSC)

DSC measurements were carried in liquid nitrogen atmosphere using DSC-822e instrument (Mettler Toledo). The calibration was made with indium as standard. Samples were weighed (2.5 ± 0.2 mg) and sealed in aluminium pans. Then, they were heated from 25° to 350 °C, at a scanning rate of 10 °C.min^{-1}. Data were treated using LAB mettler star SW 8.1 software (Mettler-Toledo International Inc, Swiss).

Contact Angle

Water contact angles of hydrogels were measured using a contact angle measurement apparatus (OCA15 Plus; Dataphysics, Germany). A water drop (3 µL) was placed over the clean hydrogel surfaces with an autopipette. All measurements were performed at room temperature.

All the characterization methods were made in triplicate for each hydrogel (gel-β, gel-β/gallic acid, gel-HPβ, gel-HPβ/gallic acid).

IN VITRO ANALYSIS OF HYDROGELS' BIOLOGICAL PROPERTIES

Antibacterial Activity

The hydrogels' antibacterial activity was tested against 3 bacteria: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296). The bacteria were grown in tryptic soy agar (TSA, Merck, Germany) for 24 h at 37 °C. The cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 18 h at 37 °C under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to 1x10^{6} cells.mL^{-1}, via absorbance readings and the corresponding calibration curve.

The hydrogels activity was measured both qualitatively and quantitatively. For the first analysis, the Disc diffusion method described by the National Committee for Clinical Laboratory Standards (NCCLS), M2-A8 document [15], was used with some modifications. The TSA was the nutritive media used and 200 µL of each inoculum (1x10^{6} cells.mL^{-1}) was spread on the media plates. The

hydrogel samples (30 ± 3 mg and with 1 ± 0.2 mm of diameter) were placed over the petri dish. The plates were then incubated at 37 °C for 24 h. The size of the inhibition halo was measured.

For the quantitative assays of the hydrogel antibacterial capacity, the samples (30 ± 3 mg) were immersed within 5 mL of $5x10^{-5}$ cells.mL^{-1} of each bacterium. Bacteria and medium controls were also included. The plates were incubated for 24 h at 37 °C. The number of viable cells, was assessed by determination of the number of colony forming units (CFUs), by plating 10 µL of cell suspension from each well onto TSA, and incubated for 24 h at 37 °C.

Both methods were made in triplicate for each bacterium in, at least, 3 independent assays.

Hydrogels Effect on Fibroblast Proliferation

The hydrogels *in vitro* cytotoxicity was carried out based on the method described on ISO 10993-5:2009 -Biological evaluation of medical devices, part 5: Tests for *in vitro* cytotoxicity, by indirect contact.

The liquid extracts of the hydrogel were prepared as follows: hydrogels (30 ± 3 mg) were immersed within 5 mL of Dulbecco's modified Eagle's medium (DMEM), and then they were kept 24 h at 25 °C in the dark.

Fibroblast 3T3 (CCL 163 - from American Type Culture Collection) were used in this study. Cells were cultured in DMEM supplemented with 10% of foetal bovine serum and 1% penicillin/streptomycin at 37 °C, 5% CO_2. After achieving confluence, cells were passed at the density of 1×10^{5} cells.mL^{-1}, using trypsin. Cells were seeded at the density of 5×10^{5} cells.mL^{-1} (48well plate) in 300 µL of DMEM complete medium.

The cytotoxicity was tested by MTS ([3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium]) assay. The medium was replaced with 300 µL of the liquid extract of hydrogels. The cells were incubated at 37 °C and 5% CO_2. After 24 h, the medium was removed and a mixture of 6 µL of MTS (Promega) and 294 µL of DMEM without phenol was added to each well. After 1 h, the absorbance value was measured at 490 nm and the results were expressed as percentage of viable cells (%), using the number of cells grown on wells without hydrogel (control +) as 100%.

All the mathematical analysis was made using the Origin Pro software.

Results and Discussion

The gallic acid encapsulation by βCD and HPβCD has been studied previous (Chapter 3.1). The ICs obtained showed good stability parameters and the gallic acid ability to destroy bacteria, usually isolated from skin and soft tissue infections, was preserved (Fig 3.1.7). Thus, these CDs were used for the CD-based hydrogel synthesis using HPMC, cellulose derivative, and cross-linked with BDGE. The HPMC is a linear polymer constituted by glycopyranose units, similar to those found on CDs. HPMC has been used within hydrogels due to its high swellability and biocompatibility [16, 17]. Hydrogels were made under alkaline pH and mild temperature as described by Lorenzo et al [6].

Attempts to prepared hydrogels based only on βCD and HPβCD (without HPMC), following the same protocol failed. Other authors [5, 7, 8] reported the formation of HPβCD hydrogels using EGDE, as cross-linking, agent within similar conditions. In the present work, BDGE was used as cross-linking agent instead of EDGE. Both molecules have 2 glycidyl groups, capable of react, simultaneous, with the CDs' hydroxyl, amino or carboxylic group or with cellulose ether group. They differ on the size of the chain between the epoxy groups, BDGE has longer chain which increase the distance between the CD and cellulose molecules [6]. Thus, it was expected to obtain a more flexible hydrogel without losing the non-toxic behaviour of the cross-linking agent [18]. However, this characteristic may, also, cause the lack of suitable mechanical properties observed on the hydrogels without HPMC.

The CD-based hydrogels were cross-linked with BDGE in the presence of HPMC and 1:1.25:0.1 ratio between compounds were maintained, since it was described as the best condition, by Rodriguez-Tenreiro et al [7]. The hydrogels obtained with HPMC (gel-β and gel-HPβ) were transparent and easily to handle, with smooth and continuous surface. They also had enough elasticity, suitable for direct contact with injured skin [6]. Additionally, the gel-β was more transparent and with lower elasticity than the gel-HPβ. After immersion in water, both hydrogels kept their shape and behaved as superabsorbent systems. All these features point out to the formation of a homogenously cross-linked hydrogels [7].

Swelling Ability

Swelling represents the water holding ability and permeability of hydrogels. The hydrogels swelling initiates with the diffusion of water molecules into the network, hydration of polar hydrophilic groups and polymer expansion until the free water molecules and the molecules within the network reach equilibrium [19, 20].

The swelling profiles (Fig 4.1.1) show that both hydrogels had a fast swelling during the first 400 min and the equilibrium was reached after 700 min. Fig 4.1.1 demonstrated that gel-HPβ was capable of taking more quantity of water molecules than the gel-β. In addition, the Q values shown that gel-HPβ absorbed 9 times more its weight in water, and the gel-β only 2.5 times. Similar swelling profiles have been reported CD- based hydrogels HPMC [5, 7, 21].

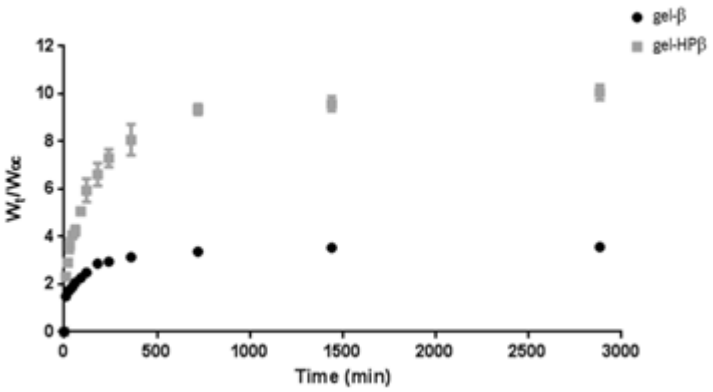

Fig 4.1.1 Swelling profiles in water of gel-β (●) and gel-HPβ (■) dry hydrogels prepared with 25% CD/ 0.25% HPMC.

Based on the swelling profiles, both hydrogels network are suitable for water and small size molecules diffusion. However, the gel-β had lower affinity to water probably related with higher degree of cross-linking or with overlapped HPMC chains within the network [6]. Additionally, the hydroxypropyl groups of HPβCD increase the network porous size, improving the network ability to absorb water molecules.

Gallic acid Loading

Hydrogels ability to retain molecules depends on (1) the network degree of cross-linking, (2) the water affinity of the polymeric molecules, and (3) interactions between the guest and the network components. All these factors regulate the molecule ability to diffuse within the hydrogel [22].

Fig 4.1.2 displays the quantity of gallic acid incorporated within hydrogels. The loading was performed with gallic acid solution dissolved in H_3PO_4/NaOH buffer at pH 3, since it was described as the best condition for the IC formation between this phenolic and βCD or HPβCD (Chapter 3.1).

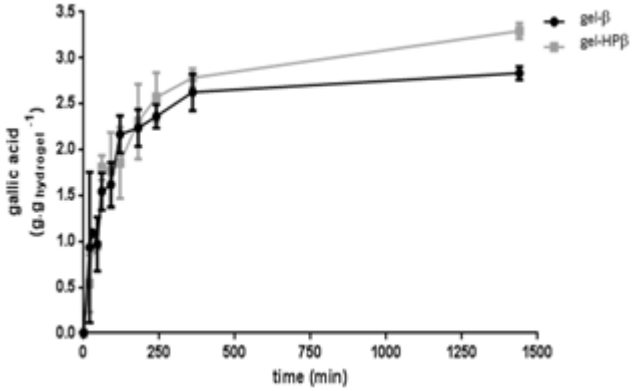

Fig 4.1.2 Gallic acid loading (a) in gel-β (black) and gel-HPβ (grey). The loading was performed during 24 h, 25 °C with gallic acid dissolved on the buffer H_3PO_4/NaOH (pH 3 ± 0.5).

The hydrogel rate of antibacterial agent incorporation was similar to the swelling profile. The equilibrium between the free gallic acid and within the hydrogel was achieved after 6h. Nevertheless, the difference between the 2 hydrogels obtained for the swelling was not so obvious in the case of gallic acid loading. Both hydrogels showed similar ability to incorporate gallic acid (gel-β 2.76 $g.g_{hydrogel}^{-1}$ and gel-HPβ 3 $g.g_{hydrogel}^{-1}$).

In the present work, both hydrogels loading capacity was similar, suggesting that the encapsulation of gallic acid by βCD and HPβCD was crucial for the hydrogel gallic acid loading. βCD and HPβCD were described as suitable for the encapsulation of poor soluble gallic acid with 1:1 stoichiometry and good stability parameters (K 40 and 90 M^{-1}) (Chapter 3.1). According with the K values published, the gel-HPβ kept the ability to form IC with higher stability than the native CD, since the gallic acid loaded was higher for this hydrogel.

HYDROGEL CHARACTERIZATION

FTIR Analysis

The hydrogel cross-linking was characterized based on the FTIR spectra of dried hydrogels and powders CDs and HPMC (Fig 4.1.3). As expected, the spectra from both CD were similar as result of their truncate shape with one edge lined with primary hydroxyl groups (OH) and the other edged with secondary groups. On their spectra, the following peaks were identified (βCD/HPβCD): 3416/3447 cm^{-1} (O-H stretching), 2924 cm^{-1} (stretching vibrational asymmetric of C-H), 1645/1640 cm^{-1} (hydrogen interactions), 1157 (C-O stretching) and 1029/1034 cm^{-1} (C-O-C) [7, 21, 23]. The HPMC spectra showed a profile similar to the CD and the strong intensity peaks were identified: 3447 cm^{-1} (O-H stretching), 2924 cm^{-1} (stretching vibrational asymmetric of C-H), 1640 cm^{-1} (hydrogen interactions), 1115 and 1063 cm^{-1} (ether bond) [24, 25].

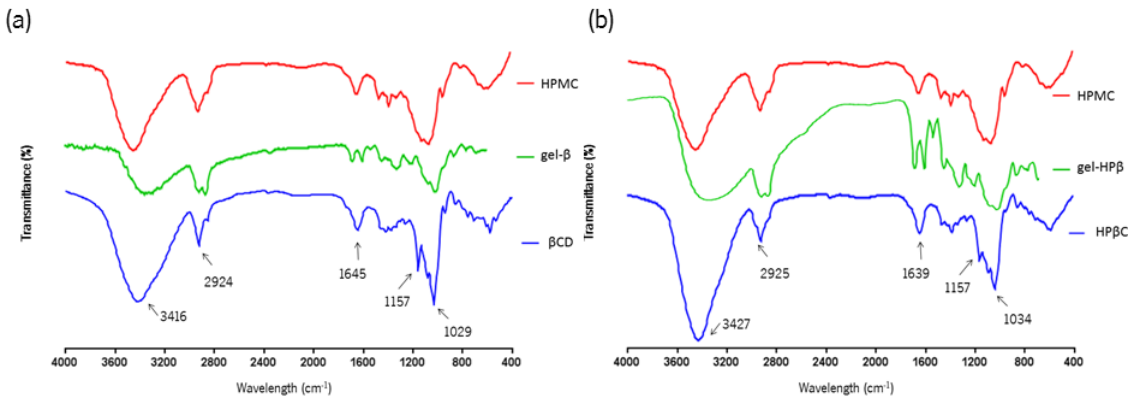

Fig 4.1.3 FTIR spectra CD (blue) and HPMC (red) powders, gel (green). (a) with βCD and (b) with HPβCD.

The hydrogels spectra had similar profile regardless the CD used for the synthesis of the polymeric network. However, they display some differences on the peaks intensity and shape when compared with the CDs and HPMC spectra. For instances, the peak attributed to the ether bonds (between 1200 and 1000 cm^{-1}) became weak and with a broad shape, as result of the decrease of those bonds induced by the cross-linking reaction. The intensity of the peaks near 3400 and 1649 cm^{-1} decreased due to the reduction of OH and hydrogen bonds, after the cross-linking [7, 21]. Thus, the majority of the CDs' OH were efficiently linked to the HPMC by the BDGE. Moreover, no peak was detected near 1250 cm^{-1} meaning that no free BDGE was within hydrogels.

Thermal Analysis

The syntheses of hydrogel induced alterations on thermal properties of each molecule (βCD, HPβCD and HPMC), as result of the interactions established to form the polymeric network. DSC thermograms displays the intermolecular interactions between hydrogels components within the network [26]. Fig 4.1.4 shows that gallic acid has a sharp endothermic peak (200 °C), as well as the 2 CDs (2 peaks near 100 °C and 275 °C). The HPMC exhibits a smaller and broad peak near 75°C. The cross-linking between HPMC and CDs induces obvious alterations on the DSC thermograms profiles. The profiles of both hydrogels (gel-βCD and gel-HPβ) have a broad peak, confirming the cross-linking between the 2 compounds, and suggesting the presence of an amorphous structure, characteristic of the hydrogel materials. Moreover, the gallic acid loaded hydrogels thermogram, also, lacks the peak of gallic acid. Thus, the gallic acid may be trapped in an amorphous or solid solution state into the polymeric network [27].

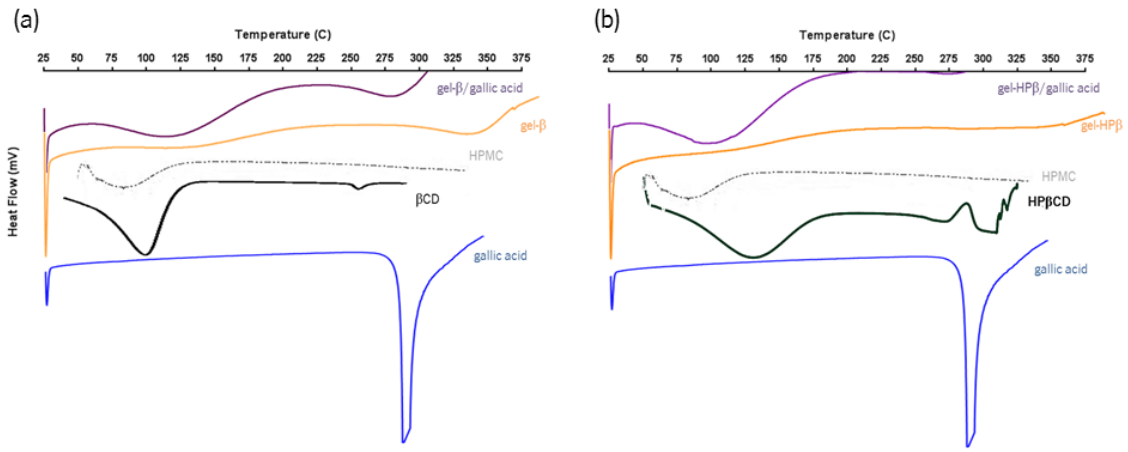

Fig 4.1.4 DSC thermograms of gallic acid (blue), CD (black), HPMC (grey), gel (orange) and gel loaded (purple). (a) with βCD and (b) with HPβCD.

Surface Hydrophilicity Measurement

The hydrogel wettability or hydrophilicity plays a major rule on biocompatibility, since the polymer surface states the interaction between the living system and the medical device [28]. This property can be determined by the measurement of the contact angle formed between water and the hydrogel. The values obtained with gel-β and gel-HPβ with or without gallic acid are on Table 4.1.1.

Table 4.1.1 Contact angle obtained with water when in contact with the hydrogels superficies

Contact angle (°)	Gel-β	Gel-HPβ
Without gallic acid	85.68±1.33	97.37±1.63
With gallic acid	73.83±1.27	106.82±2.86

Regarding gel-β, the contact angle measured was lower than 90°, thus this network has a lower hydrophilic surface [29]. Moreover, the contact angle of gel-β decreased after the gallic acid loading. An opposite behaviour was detected for the gel-HPβ. This hydrogel displayed a higher contact angle than the hydrogel with the native CD, and the gallic acid loading increased the hydrophobicity of its surface.

The hydrogel major characteristic is the ability to absorbed large amount of water, so it should be expected to obtained low contact angles, due to hydrogel hydrophilic surface. However, the macromolecules within the networks hold high mobility to rearrange their orientation according to the environment. Thus, in the specific case of hydrogels, the contact angle value reflects the degree of freedom of the network molecules, to move as response to the environment change [30].

According to the swelling and gallic acid loading results (Fig 4.1.1 and 4.1.2), it was predictable that the gel-HPβ had a lower contact angle. The apparent hydrophobicity observed for this hydrogel surface may be caused by the less freedom of the network to rearranged their macromolecules, due to the hydroxypropyl groups [31]. These groups increase the molecules size which enlarge the mesh size (higher swelling), but decrease the molecules mobility within the network (low hydrophilic surface). Moreover, the HPβCD has the ability to establish an IC with gallic acid with higher stability parameters than the βCD (Chapter 3.1). Thus, the gallic acid interaction with this cyclodextrin will be stronger, contributing to the minor interaction of the phenolic with water, and consequently the degree on hydrophilicity of gel-HPβ network.

Gallic acid Release from Hydrogels

Fig 4.1.5 displays the gallic acid release profiles in SSS; this solution was chosen in order to simulate the behaviour of the hydrogels in contact with the skin. Both hydrogels were capable of control the release for 2 days, time at which the equilibrium between the gallic acid within the

network and on solution was achieved. Additionally, the release profiles were also analogous for the 2 networks, there was a burst release during the first 6h, as result of gallic acid migration from the hydrogels surface to the solution. The slower release, after 6h, can be ascribed to the phenolic compound dissociation from the CDs, followed by its diffusion through the network. Moreover, the amount of gallic acid released was higher for the gel-HPβ, as result of the greater swelling capacity of this network, as referred above.

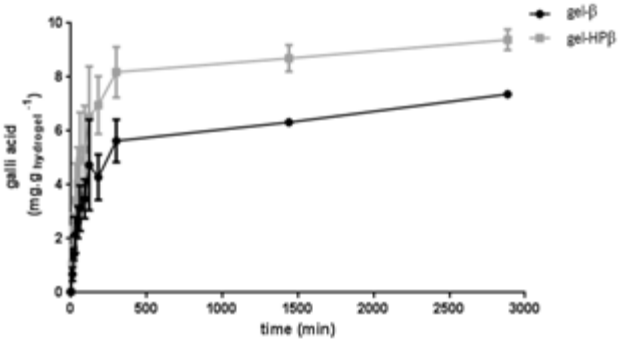

Fig 4.1.5 Gallic acid release from gel-β (black) and gel-HPβ (grey) hydrogels, it was performed during 48 h, 25 °C within synthetic sweat solution (pH 5 ± 0.1).

BIOLOGICAL ACTIVITY OF HYDROGELS

Antibacterial Activity

Gallic acid antibacterial activity has been attributed to its interaction with cells' surfaces (enhanced by gallic acid affinity to the lipophilic membrane layer), which induce alterations on the cell electrochemical potential, and reduce the membrane integrity. The gallic acid uptake induce a hyperacidification of cytoplasm, via proton donation, interfering with crucial metabolic pathways [32]. Previous research reported that gallic acid antibacterial activity was conserved after encapsulation by βCD and HPβCD (Chapter 3.1).

The main goal of the present work was to developed a hydrogel capable of prevent wound infections. Hence, the antibacterial activity of the polymeric networks was evaluated by qualitative and quantitative methods. Fig 4.1.6 shows the growth inhibition halos obtained for the gallic acid loaded hydrogels. The gallic acid maintained its ability to reduce the bacteria growth (halos between 3.5 and 2.7 mm).

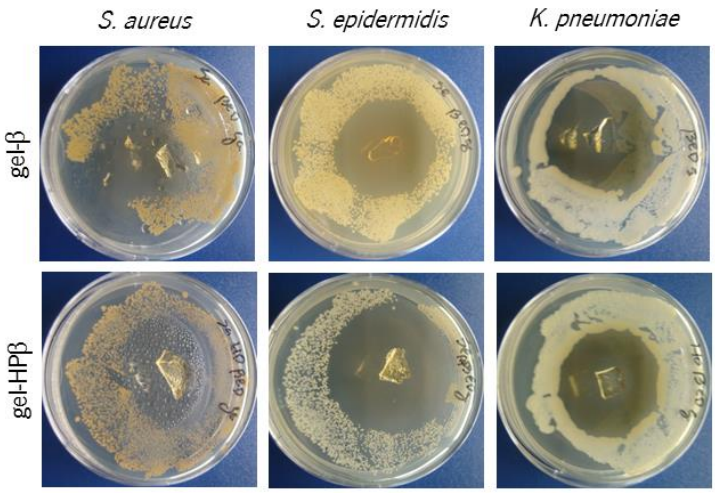

Fig 4.1.6 Qualitative analysis of the gallic acid load hydrogels antibacterial activity against 3 bacteria (*K. pneumoniae*, *S. epidermidis* and *S. aureus*, $1x10^5$ cel.mL^{-1}).

The quantification of the hydrogels' antibacterial activity, against the 3 bacteria (Fig 4.1.7), was consistent with the previous analysis (Fig 4.1.6). The gel-β and gel-HPβ were capable of destroy all the bacterial cells, with the exception of the gel-HPβ when in contact with *S. aureus.* Although, in this case the growth observed was minimum (only 1 log).

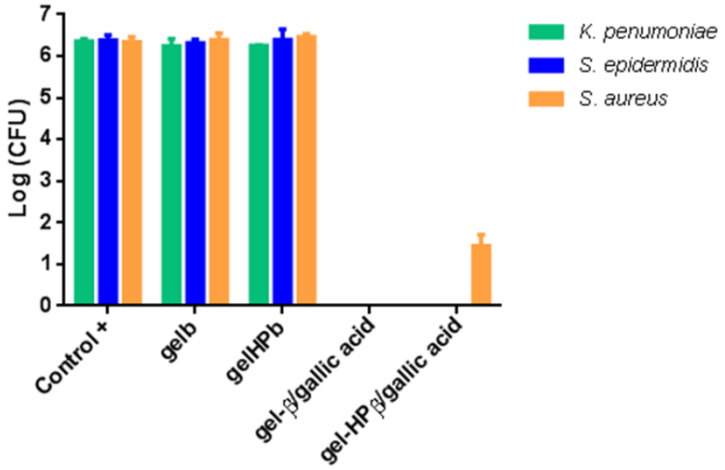

Fig 4.1.7 Quantitative analysis antibacterial activity of the gallic acid load hydrogels by direct contact against 3 bacteria (*K. pneumoniae*, *S. epidermidis* and *S. aureus*, $5x10^5$ cel.mL^{-1}). All data is expressed as mean + standard deviation (n = 9).

Therefore, it was verified that the gallic acid antibacterial activity was preserved after incorporation within the hydrogels. Considering that the hydrogels samples had 30 mg, and the gallic acid release (gel-β 7.4 $mg.g_{hydrogel}^{-1}$ and gel-HPβ 9.4 $mg.g_{hydrogel}^{-1}$, Fig 4.1.5), the concentration of gallic acid in contact with bacteria would be, at least, 0.26 mM for gel-β and for gel-HPβ 0.33mM. These values are lower than MBC reported (0.47 mM) (Chapter 2 and 3.1). Despite that, both networks were capable significantly decreased the 3 bacteria growth. Thus, antibacterial activity of hydrogels was based, not only on the amount of phenolic capable of migrate from the hydrogel to the solution, but also on the gallic acid tramped inside the hydrogels.

Therefore, the gallic acid was successfully loaded into the polymeric network produced, and its release was sustained for 48 h. Moreover, the loaded gel-β and gel-HPβ were capable of destroy all the bacteria cells preserving the gallic acid antibacterial activity.

Effect of Hydrogels on Fibroblast Proliferation

The successfully utilization of gel-β and gel-HPβ as wound dressing, also, depends on their biocompatibility. Biocompatibility reflects the interaction between the artificial material and tissues, and it can be evaluated by *in vitro* cytotoxicity [28]. In the present study, MTS test was used to evaluate the cytotoxicity effect of the new developed hydrogels. The MTS assay allowed the measurement of the cellular viability, due to cell capacity to uptake MTS and its subsequent reduction by the mitochondria, leading to alterations on MTS colour.

The percentage of viable cells (Fig 4.1.8) measured after contact with the extracts from gel-β and gel-HPβ were similar to the percentage of the normal conditions of growth (control +). Thus, both hydrogels do not release any kind of substance that could be potentially hazardous for fibroblast.

The gallic acid induces a reduction on the viable cells (Fig 4.1.8). It was previously shown that gallic acid can enhance fibroblasts proliferation when applied at low concentrations, and induces a reduction on viable cells for concentrations above 0.6 mM (Chapter 2). Assuming that, at least, 0.26 or 0.33 mM of gallic acid were released to the medium used for contact with fibroblast, the gallic acid effect on fibroblast proliferation was similar to the one described for the free gallic acid.

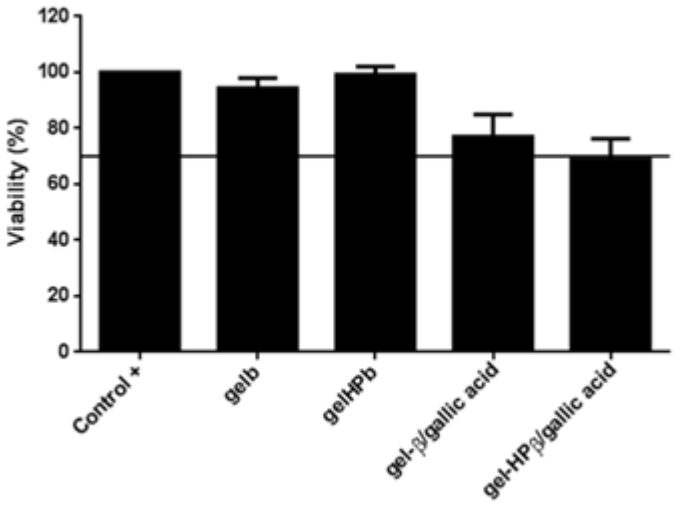

Fig 4.1.8 Viability of fibroblast 3T3 after 24 h of contact with liquid extracts from hydrogels (24 h within DMEM), measured with MTS assay. The control + allowed the perfect growth of the cells. All data is expressed as mean + standard deviation (n = 9).The line indicate 70% of cell viability.

Thus, the phenolic incorporation within both hydrogels did not changes the gallic acid biological properties. Moreover, the percentage of viable cells, for both hydrogels, was above the limit described as safety for humans (70%), based on ISO 10993-5:2006.

Therefore, gel-β and gel-HPβ conjugated with gallic acid may be a viable option for wound dressing without causing any damage to the surrounding tissue.

Conclusion

The CD-based hydrogels obtained from the cross-linking between βCD or HPβCD and HPMC were developed in order to be applied as wound dressing capable of preventing wound infections. To best of authors' knowledge, loading and release of gallic acid (as antibacterial agent) into hydrophilic networks of CD, HPMC and BDGE (as cross-linking), and its release for control wound infections, have not been evaluated until know.

The hydrogels obtained after successful cross-linking with BDGE, under mild conditions were transparent, easy to handle and soft, thus suitable for the contacts with injury skin. Gel-β and gel-HPβ behaved as superabsorbent hydrogels, being the last network capable of higher swelling. The swelling and gallic acid loading profiles were similar. The results obtained (DSC, FTIR and loading) suggested that gallic acid may be inside the cyclodextrins cavity and, also, trapped in the polymeric network. The gel-HPβ has a less hydrophilic surface when compared with the gel-β, as result of the lower mobility of their network. Regarding the biological properties of both hydrogels, the gallic acid antibacterial activity was preserved after its incorporation within the hydrogels. In addition, all the hydrogels, with or without gallic acid, enabled the fibroblast proliferation.

In conclusion, the gallic acid was successfully loaded into the polymeric network produced, and its release was sustained for 48 h. Moreover, the load gel-β and gel-HPβ were capable of destroying bacterial cells preserving the gallic acid antibacterial activity. Based on the results from the present work, the gel-HPβ appears to be the network with more suitable properties for the incorporation of gallic acid and utilization as antibacterial wound dressing, without causing any damage to the surrounding tissue.

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Chapter 4.2

Caffeic acid loading wound dressing: physicochemical and biological characterization

THE PRESENT CHAPTER WAS ADAPTED FROM THE FOLLOWING PAPER

Pinho E, Soares GMB, Henriques M. Caffeic acid loading wound dressing: physicochemical and biological characterization.
Future Microbiology (Submitted).

Abstract

Caffeic acid, a natural phenolic compound, has been described has active against bacteria common isolated from wound infections. However, its low stability under environmental stress and poor solubility reduced caffeic acid applicability as pharmaceutical product. However, these parameters can be enhanced by complexation with cyclodextrin. So, the main goal of the present work was to incorporate caffeic acid on cyclodextrin-based hydrogels capable of control delivery in order to be used as antibacterial wound dressing.

Cyclodextrins-based hydrogels were prepared by direct cross-linking of β-cyclodextrin (gel-β) or hydroxypropyl-β-cyclodextrin (gel-HPβ) with 1,4-butanediol diglycidyl ether (BDGE) in the presence of hydroxypropyl methylcellulose (HPMC). The polymeric networks were transparent and viscoelastic. Both hydrogels were capable of load and release caffeic acid. However, gel-β showed better loading capacity and lower release, as expected due to β-cyclodextrin higher affinity to form inclusion complexes with caffeic acid.

The caffeic acid loaded hydrogels were effective against *Klebsiela pneumoniae, Staphylococcus epidermidis* and *Staphylococcus aureus*. Moreover, the hydrogel interaction with fibroblasts was better for the gel-β.

The hydrogels obtained combine good physicochemical properties (viscoelasticity, superabsorbency and high ability to retain and deliver caffeic acid) with the preservation of caffeic acid antibacterial activity, being the gel-β the most suitable. Thus, this hydrogels could be useful as caffeic acid delivery system's device for the treatment of wound infections.

Keywords: Antibacterial activity, caffeic acid, cellulose, cyclodextrin, hydrogel, wound dressing.

Introduction

Hydrogels have been successfully applied as medical devices, special for wound healing purposes [1]. Wound dressing hydrogels are polymeric networks with a three-dimensional structure capable of absorbing high amounts of water. These materials, besides mechanical protection, may enhance the healing process by promoting gas exchange, and reduce the body fluids loss. The biological properties of hydrogels can be improved by the absorption of bioactive molecules on their network, such as growth factors to accelerate the healing process or antimicrobial agents to prevent infections [2]. However, hydrogels have poor properties regarding the controlled release of those bioactive molecules. Thus, cyclodextrins (CD), a truncated oligosaccharide with ability to complex with a wide range of molecules, have been used to enhance the hydrogels drug delivery ability. CD-based hydrogels retained the suitable properties of polymeric networks (swelling, softness and mechanical properties) and, additionally, the capacity of CD to complex and sustainably release bioactive molecules [3].

Polyphenolics are extensive distributed among plant kingdom, imposing plant odours, pigmentation and flavour or/and acting as plant defence mechanism, against tissue infections or injuries [4, 5]. These plants' metabolites are present in human diet, and a wide range of biological effects have been attribute to them, like anti-oxidant, anti-inflammatory, antimicrobial and antiviral [6–8]. Polyphenolics, the term applied for the set of molecules that share a common chemical skeleton (one or more aromatic ring with at least one hydroxyl group (OH) attached), but with different structures and functions [9].

Caffeic acid (3,4-dihydroxycinnamic acid) is a simple phenolic acid, and has been reported as anti-oxidant, antibacterial and fungicide [10–12]. This phenolic acid, as well as other polyphenolics, have been used as food preservative and proposed as antimicrobial agent for the pharmaceutical field. Though, caffeic acid exhibits poor solubility and stability under environmental stress, reducing its applicability as antibacterial agent on pharmaceutical field. Thus, encapsulation devices, such as cyclodextrins, have been suggested to ensure caffeic acid stability and improved its utilization as antibacterial agent (Chapter 3.2).

The main goal of the present work was to develop a hydrogel wound dressing based on cellulose and cyclodextrins, with the ability to retain and sustain the release of caffeic acid, for wound dressing proposes.

Material and methods

Material

Caffeic acid (3,4-dihydroxycinnamic acid) was purchased from Sigma, β-cyclodextrin (βCD, 1135 $g.mol^{-1}$) and 2-hydroxypropyl-β-cyclodextrin (HPβCD, 1309 $g.mol^{-1}$) were acquired from AppliChem. Hydroxypropyl methylcellulose (HPMC) Methocel® K4M was purchased from VWR Prolab and 1,4-butanediol diglycidyl ether (BDGE), 50-60% in water, was purchased from Acros Organics. All solutions were prepared with purified water, obtained by reverse osmosis (MiliQ®, Mikipore, Madrid, Spain) with a resistivity above 18.2 $M\Omega.cm^{-1}$.

Synthesis of gel-β and gel-HPβ

Hydrogels were synthesized through the method described by Lorenzo et al [13]. Briefly, 2.5 g of each CD (βCD or HPβCD) was dissolved in 10 mL of NaOH (0.2 M). The solutions were maintained for 5 min, at 25 ºC and under mechanical agitation (200 rpm). After, 0.025 g of HPMC was added to each solution, and the solubilisation was improved by mechanical agitation (200 rpm) during 5 min, at 25 ºC. The cross-linking agent (BDGE, 2 mL) was added to 5 mL portions of each solution within petri dishes. The petri dishes were sealed with parafilm and kept for 2 min at 25 ºC, and under mechanical agitation (200 rpm). To complete the cross-linking process, the plates were preserved at 50 ºC for 12 h. At this temperature, the CD and HPMC stability was assured. After cooling, hydrogel were immersed in ultrapure water for 12 h and 25 ºC, to allow their swelling. Then, the hydrogels were transferred for HCl solution (10 mM) for more 12 h, and finally immersed in water for 7 days.

The dry process was performed as followed: the hydrogels were kept at 25 ºC for 24 h and, after, were transferred to a desiccator until the weight stabilizing.

At the end two hydrogels were obtained: βCD-co-HPMC (gel-β) and HPβCD-co-HPMC (gel-HPβ).

Preparation and Characterization of Caffeic acid Load

For the hydrogels loading, a caffeic acid solution ($2.3x10^{-2}$ M, 2% ethanol) was prepared using H_3PO_4/NaOH buffer (pH 5 ± 0.5). To assurance that the caffeic acid was completely dissolved,

the solution was maintained at 50 °C, 200 rpm for 30 min. Dry hydrogels samples (90 ± 3 mg) were immersed in 5 mL of caffeic acid solution, 25 °C and 60 rpm. The amount of phenolic acid in the solution was assessed by UV-Vis spectrophotometry, until the absorbance values stabilized. The caffeic loading was calculated based on the variation of the phenolic acid on the initial solution and on the equilibrium. The absorption spectra were measured in the range 200–360 nm and recorded on a Jasco V560 spectrometer, using a 1cm quartz cuvette.

The hydrogels with and without caffeic acid were characterized by differential scanning calorimetry (DSC), Fourier transform infrared spectroscopy (FTIR) and contact angle.

DSC measurements were carried in liquid nitrogen atmosphere using DSC-822e instrument (Mettler Toledo). The calibration was made with indium as standard. Samples were weighed (2.5 ± 0.2 mg) and sealed in aluminium pans. Then, they were heated from 25 to 350 °C at a scanning rate of 10 °C.min^{-1}. Data were treated using LAB mettler star SW 8.1 software (Mettler-Toledo International Inc, Swiss).

The FTIR-ATR analysis was performed as follows: samples (0.5 ± 0.05 g) of each hydrogel were used. The IR spectra were recorded between 400 and 4000 cm^{-1}, in Avatar 360 FTIR spectrometer. The spectra of compounds alone (caffeic acid, βCD, HPβCD and HPMC) were, also, recorded, using the potassium bromide pellet technique.

Surface contact angles of hydrogels in contact with ultrapure water were measured using a contact angle measurement apparatus (OCA15 Plus; Dataphysics, Germany). A 3 µL water drop was placed over clean hydrogel surface with an autopipette. All measurements were performed at room temperature.

Hydrogels characterization was made in triplicate for each hydrogel (gel-β, gel-β/caffeic acid, gel-HPβ, gel-HPβ/caffeic acid).

In vitro Caffeic acid Release

Dry load hydrogels (90 ± 3 mg) were immersed in 5 mL of synthetic sweat solution (SSS, 0.5 g *L*-histidine monohydrochloride monohydrate, 5 g of sodium chloride, 2.2 g of sodium dihydrogen orthophosphate dihydrate, pH 5 ± 0.5), at 25 °C. At predetermined time points, samples were taken until equilibrium was achieved. The amount of caffeic acid release was measured by the

UV-Vis spectrophotometry, within a range 200–360 nm and recorded on a Jasco V560 spectrometer, using a 1cm quartz cuvette.

Antibacterial Assessment

The hydrogels antibacterial activity was tested against 3 bacteria: *Staphylococcus epidermidis* (ATCC 12228), *Staphylococcus aureus* (ATCC 6538) and *Klebsiella pneumoniae* (ATCC 11296). The bacteria were grown in tryptic soy agar (TSA, Merck, Germany) for 24 h at 37 ºC. Then, the cells were inoculated in tryptic soy broth (TSB, Merck, Germany) and incubated for 18 h at 37 ºC under agitation (120 rpm). Subsequently, bacterial concentration of each strain was adjusted to $5x10^5$ cells.mL^{-1}, via absorbance readings and corresponding calibration curve.

For the quantitative assess of hydrogel antibacterial capacity, samples with 90 ± 3 mg were immersed within 5 mL of $5x10^5$ cells.mL^{-1} of each bacterium. Bacteria and medium controls were, also, included. The hydrogels and the bacteria were incubated for 24 h at 37 ºC. The number of viable cells was assessed by determination of the number of colony forming units (CFUs), plating 10 µL of cell suspension from each replicates onto TSA, and incubated for 24 h at 37 ºC.

The assays were made in triplicate for each bacterium and hydrogel combination, at least in 3 independent assays.

Cytotoxicity Assay

The hydrogels *in vitro* cytotoxicity was carried out based on the method described on ISO 10993-5:2009 -Biological evaluation of medical devices, part 5: Tests for *in vitro* cytotoxicity, by indirect contact. The liquid extracts of the hydrogel were prepared as follows: hydrogels (90 ± 3 mg) were immersed within 5 mL of Dulbecco's modified Eagle's medium (DMEM), and then they were kept 24 h at 25 ºC, on dark.

Fibroblast 3T3 (CCL 163), from American Type Culture Collection, were used in this study. Cells were cultured in DMEM supplemented with 10% of foetal bovine serum and 1% penicillin/ streptomycin at 37 ºC, 5% CO_2. After achieving confluence, cells were passed at the density of 1×10^5 cells.mL^{-1}, using trypsin. Then, cells were seeded at the density of 5×10^5 cells.mL^{-1} (48 well plate) in 300 µL of DMEM complete medium. The 48 well plates were incubated for 24 h, 37 ºC

and 5% CO_2 and after, the medium was replaced for 300 µL of each hydrogels extract and incubated for more 24 h.

The cytotoxicity was tested by MTS ([3-(4,5-carboxymethoxyphenyl)-2-(4-sulfophenyl)- 2H-tetrazolium]) assay. This assay measured the cells viability by their capacity to uptake MTS and subsequent reduction by mitochondria, leading to the coloration of MTS. Thus, the liquid extract of hydrogels was removed, and a mixture of 6 µL of MTS (Promega) and 294 µL of DMEM, without phenol, was added to each well. After 1 h, the absorbance value was measured, at 490 nm, and the results were expressed as percentage of viable cells (%), using the number of cells grown on wells without hydrogel (control +) as 100%.

Statistical Analysis

Statistical analysis was performed with OriginPro 8 SR0 (V8.70724, OriginLab Corporation). The differences between groups were evaluated using the 2-way ANOVA variation test and the results were considered statistically significant for p values < 0.0001.

Results

Caffeic acid Loaded gel-β and gel-HPβ

Fig 4.2.1 displays the caffeic acid loading profiles of gel-β and gel-HPβ. The hydrogels obtained were capable of absorbing caffeic acid and achieving the equilibrium after 360 min. Interestingly, gel-β was capable of load a higher amount of caffeic acid (0.6 $g.g_{hydrogel}^{-1}$) when compared with the gel-HPβ (0.37 $g.g_{hydrogel}^{-1}$).

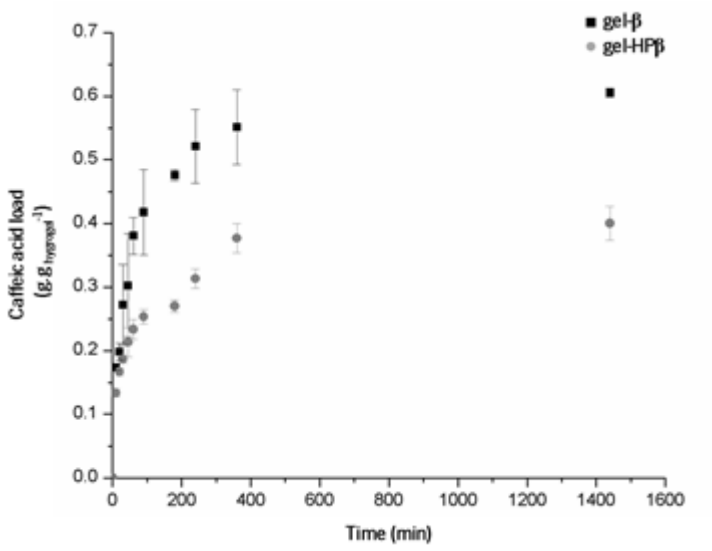

Fig 4.2.1 Caffeic acid loading (a) in gel-β (black) and gel-HPβ (grey). The loading was performed during 24 h, 25 °C with caffeic acid dissolved on the buffer H_3PO_4/NaOH (pH 5 ± 0.1, 2% ethanol).

Physicochemical Characterization of gel-β and gel-HPβ

The hydrogels with or without caffeic acid were characterized by DSC, FTIR and surface hydrophilicity.

The DSC thermograms of various products are shown in Fig 4.2.2. The DSC curve of caffeic acid display a single sharp endothermic peak near 225 °C, corresponding to its melting point. The CD (βCD and HPβCD), also, showed 2 defined peaks each, near 100 °C and 275 °C. In the case of HPMC, owing to its amorphous nature, a broad endothermic peak was observed at about 75 °C. The hydrogels formation and cross-linking could be confirmed by the lack of obvious peak on their thermograms (orange line). Moreover, a nonobvious peak was obtained for loaded hydrogels.

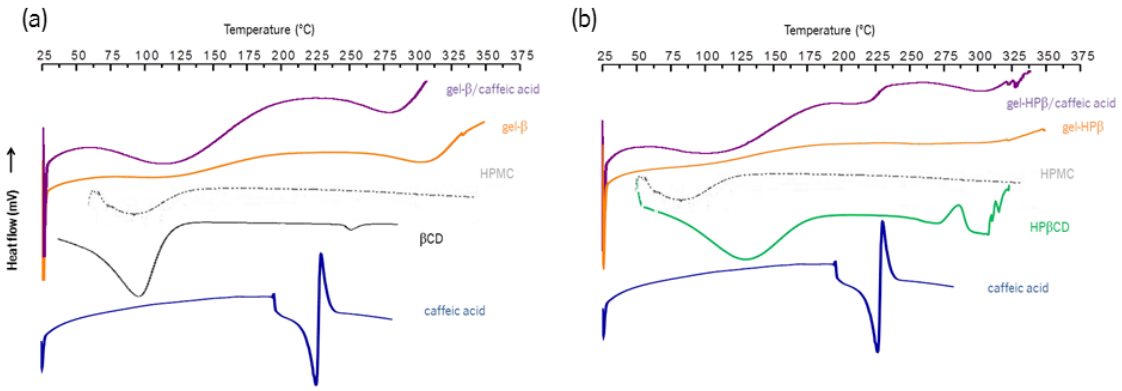

Fig 4.2.2 DSC thermograms of caffeic acid (blue), CD (black), HPMC (grey), gel (orange) and gel loaded (purple). (a) with βCD and (b) with HPβCD.

Fig 4.2.3 present the FTIR spectra obtained from the gel-β and gel-HPβ with and without caffeic acid, and also, the network components alone (βCD, HPβCD, HPMC and caffeic acid). Both CD spectra shows higher intensity on the following peaks (βCD/HPβCD): 3416/3447 cm^{-1} (O-H stretching), 2924 cm^{-1} (asymmetric vibrational stretching of C-H), 1645/1640 cm^{-1} (hydrogen interactions), 1157 cm^{-1} (C-O stretching) and 1029/1034 cm^{-1} (C-O-C) [3, 14, 15]. The HPMC spectrum showed a profile similar to the CD and the strong intensity peaks were identified: 3447 cm^{-1} (O-H stretching), 2924 cm^{-1} (asymmetric vibrational stretching of C-H), 1640 cm^{-1} (hydrogen interactions), 1115 and 1063 cm^{-1} (ether bond) [16, 17].

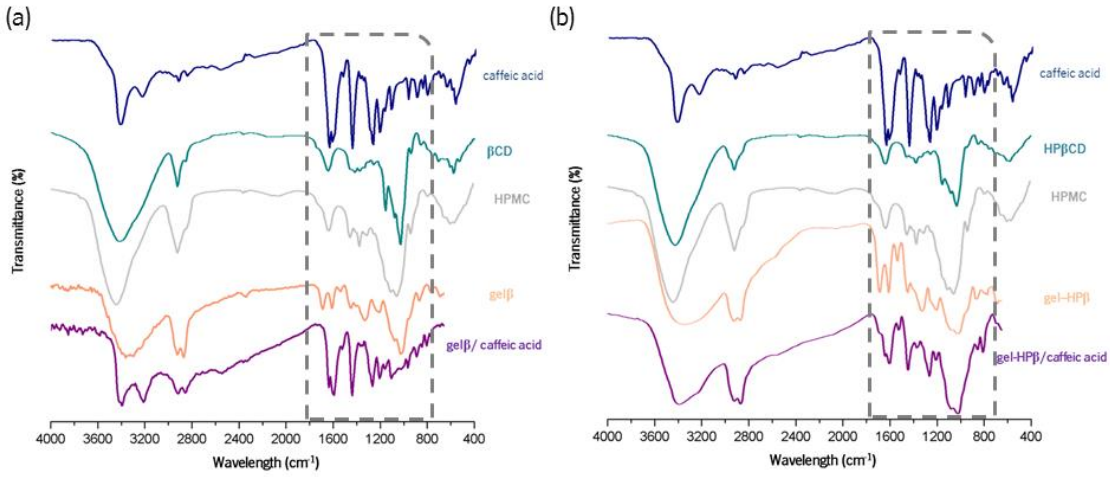

Fig 4.2.3 FTIR spectra of caffeic acid (blue), CD (green), HPMC (grey), gel (pink) and gel loaded (purple). (a) with βCD and (b) with HPβCD.

The hydrogels spectra had similar profile regardless the CD used for the synthesis of the polymeric network. However, they display some differences on the peaks intensity and shape when compared with the CDs and HPMC spectra. The changes observed in the region of ether bond signals (between 1200 to 1000 cm^{-1}), and the decrease of peaks intensity at 3400 and 1649 cm^{-1} confirms that cross-linking occurred during the process.

The spectra obtained for the load gel-HPβ was similar to the gel without caffeic acid. However, the phenolic incorporation within the gel-β induced some changes on the spectra, more obvious between 2000 and 800 cm^{-1}. In this range, the caffeic acid peaks were present on the load gel-β spectra, making it more alike to the phenolic spectra than to the hydrogel.

The hydrophilicity of the hydrogels surface was assessed by the contact angle formed after a water drop was released on the materials surface. The gel-β had a contact angle of 85.68 ± 1.33 and the gel-HPβ 97.37 ± 1.63. So, both surfaces can be considered hydrophilic, being the gel-β more suitable for contact with water [18]. The caffeic acid loading slightly reduced the hydrophilicity of the gel-β (78.73 ± 1.69), and had no influence on the surface properties of the gel-HPβ (97.82 ± 1.68).

In vitro Caffeic acid Release

Both polymeric networks were capable of sustain the caffeic acid release for 48 h (Fig 4.2.4), with a burst at the first 60 min and a slower release until the equilibrium (360 min). After 2 days, the gel-HPβ released 1.2 $mg.g_{hydrogel}^{-1}$ of caffeic acid and the gel-β near half of that amount (0.77 $mg.g_{hydrogel}^{-1}$).

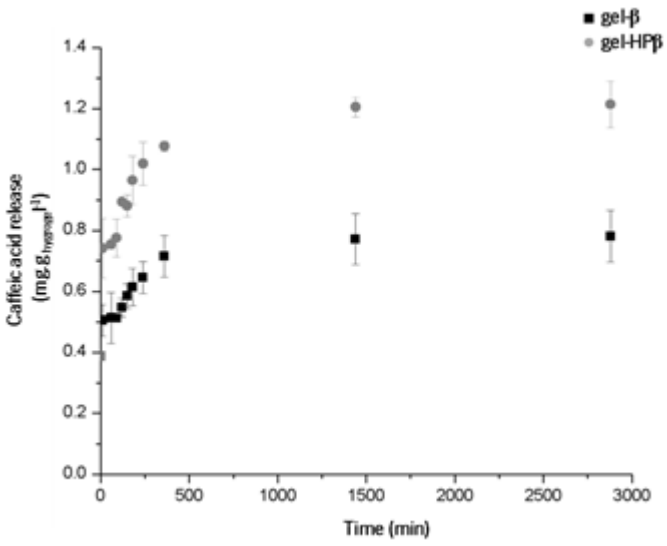

Fig 4.2.4 Caffeic acid released from gel-β (black) and gel-HPβ (grey) hydrogels; it was performed during 48 h, 25 °C within synthetic sweat solution (pH 5 ± 0.1).

Antibacterial Activity of gel-β and gel-HPβ

The hydrogels antibacterial activity evaluation revelled that both networks has comparable effects on 3 bacteria growth (Fig 4.2.5). The gel-β and gel-HPβ without caffeic acid allowed the normal growth of the cells (near 6 log), and the hydrogels with caffeic acid caused a reduction of more than 3 log on the number of viable cells, when compared with the control. Apparently the *S. epidermidis* was more susceptible to the antibacterial activity of both materials.

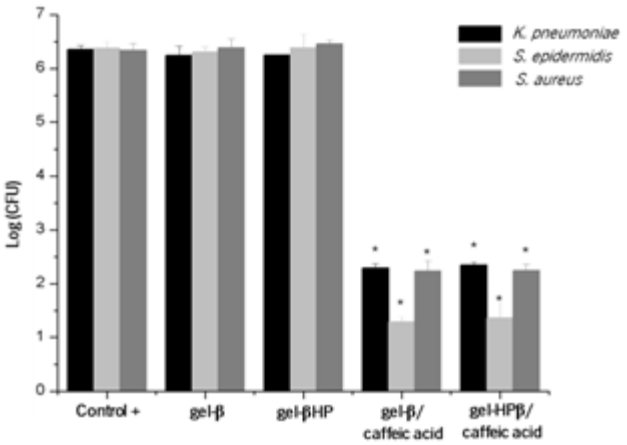

Fig 4.2.5 Antibacterial assessment of caffeic acid load hydrogels (90 ± 3 mg) by direct contact with the 3 bacteria (*K. pneumoniae*, *S. epidermidis* and *S. aureus*, $5x10^5$ cel.mL^{-1}). The control + allowed the perfect growth of the cells. All data is expressed as mean + standard deviation (n = 9). *statistically different from the control +, $p < 0.0001$.

Effect of Load Hydrogels on Fibroblasts Proliferation

Fig 4.2.6 shows the hydrogels effect on the proliferation of 3T3 fibroblast. The gel-β and gel-HPβ allowed the perfect proliferation of the fibroblast. However, the caffeic acid loading induced a reduction on the viable of cells, gel-β/caffeic acid showed a decrease of 26% on viable cells and, only, 40% of viable cells were detected regarding the loaded gel-HPβ.

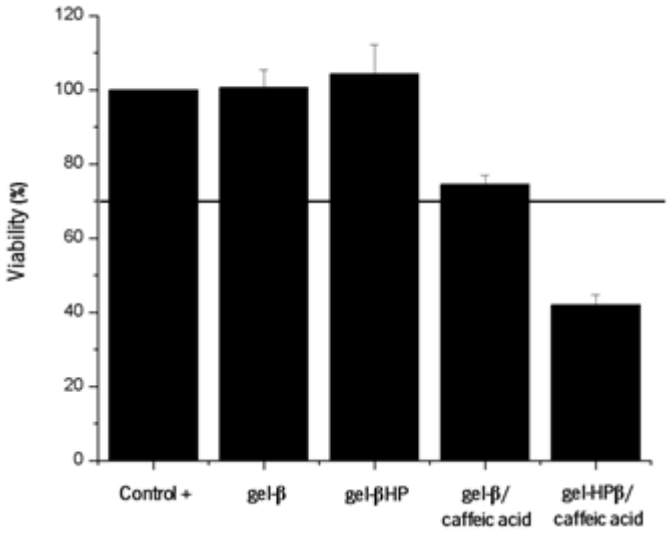

Fig 4.2.6 The viability of fibroblast 3T3 after 24 h of contact with liquid extracts from hydrogels (24 h within DMEM), measured with MTS assay. The control + allowed the perfect growth of the cells. All data is expressed as mean + standard deviation (n = 9).The line indicate 70% of cell viability.

Discussion

Physicochemical Characterization of gel-β and gel-HPβ

Previous work demonstrated that hydrogels could be obtained from the cross-linking of HPMC and βCD or HPβCD, using BDGE. The hydrogel formation occurred after 12h when a ratio of 1 CD: 1.25 BDGE: 0.01 HPMC was used (Chapter 4.1). The CD-based hydrogels obtained showed suitable characteristics for contact with injury skin. They were resistance but viscoelastic and with a smooth and continues surface. Moreover, both polymeric networks behaved as superabsorbent hydrogels and kept their shape after swelling (Chapter 4.1). Thus, the same conditions were used in the present work.

The hydrogels loading properties result from the combination of several factors, such as network cross-linking degree, water affinity to the components (swelling) and interactions between guest and the network [19]. Additionally, CD-based hydrogels ability to retain the guest molecule is, also, regulated by the formation of inclusion complexes (IC) between the CDs and bioactive molecules. Thus, the guest molecule will be present trapped in the polymeric network, and inside the CDs cavity [14, 20].

The caffeic acid loading capacity was dependent on the CD used for the hydrogel synthesis. Taking in account the swelling results (gel-HPβ > gel-β (Chapter 4.1)) it was expectable that gel-HPβ was capable of retaining higher amount of caffeic acid in the aqueous phase. However, the gel-β absorbed more than 2 fold of caffeic acid, when compared to gel-HPβ. The interaction between caffeic acid and βCD or HPβCD was analysed in a previous work. The native CD was reported to be the most suitable for the complexation with caffeic acid, at pH 5 (Chapter 3.2). Thus, the loading results and the inclusion complexes stability (caffeic acid had higher affinity to βCD than HPβCD) suggests that CDs play a major role on the hydrogels ability to load caffeic acid, and the βCD capacity to encapsulate caffeic acid was preserved after hydrogel synthesis (Fig 4.2.1).

The loading of larger molecules (diclofenac, ciprofloxacin, 3-methylbenzoic and estradiol) by similar hydrogels has been reported. All works stated that the inclusion complex, between the guest and the CDs, had a major contribution for the retention ability of CD-based hydrogels, which are in agreement with our results. Although, interactions between the guest molecule and HPMC were also referred, but with lower influence on the loading mechanism [3, 15, 20–22].

The cross-linking between CDs and HPMC, as well as the presence of caffeic acid on the hydrogels network were confirmed by the DSC and the FTIR analysis. The thermograms profiles of both hydrogels (gel-βCD and gel-HPβ) have a broad peak, as result of the cross-linking between the CD and HPMC. This suggested the presence of an amorphous structure, characteristics of hydrogels materials (Fig 4.2.2). Moreover, no significantly differences were detected between gel-βCD and gel-HPβ thermograms, thus the cross-linking process was similar regardless the CD. The thermograms of the load hydrogels (gel-β and gel-HPβ) lack the characteristic peak of caffeic acid (Fig 4.2.2). Therefore, the phenolic may be trapped in an amorphous or solid solution state into the polymeric network [23].

Regarding the FTIR spectra (Fig 4.2.3), the alterations on the peaks intensity and shape, indicating that the polymeric network had less OH and hydrogen bonds, as consequence of the cross-linking reaction [3, 14]. Moreover, the differences between the loaded gel-β and gel-HPβ spectra may result from the presence of caffeic acid in different state on the polymeric networks. In the case of the hydrogel synthetized with HPβCD, the caffeic acid is probably adsorbed in the surface, since the spectra from gel-HPβ and gel-HPβ/caffeic acid had similar profile. In opposition, the profile of gel-β and caffeic acid/gel-β had visible alterations indicating that the phenolic compound may be within the network, and interacting with the HPMC and/or βCD molecules.

Based on the results obtained, a reaction mechanism is proposed and illustrated on Fig 4.2.7. The BDGE has 2 epoxide groups able to react with the OH from CDs and HPMC, at alkaline pH (condition used), leading to the formation of stable ethers bonds. Thus, the decrease of the OH detected, on the FTIR results, were a consequence of their consumption for cross-linking reaction.

Furthermore, the physicochemical differences detected between the 2 hydrogels synthetize (gel-β and gel-HPβ) may arise from the CD' OH group involved in the reaction with BDGE. The cyclodextrins have a truncated cone shape (Fig 4.2.8) with 21 OH on the case of βCD, and HPβCD has 21 hydroxypropyl and/or OH. These groups have different reactivity, as demonstrated on Fig 4.2.8. Regarding βCD, the OH-2 and OH-3 (located on the wider side of the rim) are less reactive than the OH-6 (placed on the narrower side and directed away of the cavity), and the reactivity of the later is improved by alkaline conditions. Moreover, the HPβCD has OH on their substituents with the same reactivity than the OH-6 [3].

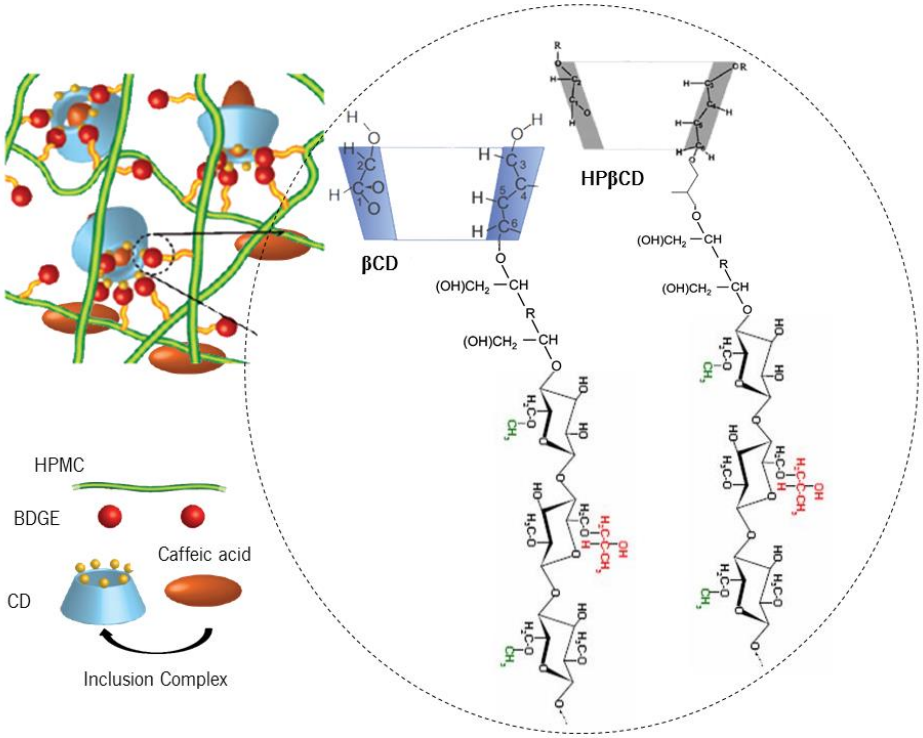

Fig 4.2.7 Schematic representation of the reaction mechanism between the cyclodextrins (βCD or HPβCD) and HPMC using BDGE, as cross-linking agent. The caffeic acid position within the cyclodextrins cavities and adsorbed on the polymeric network were also represented.

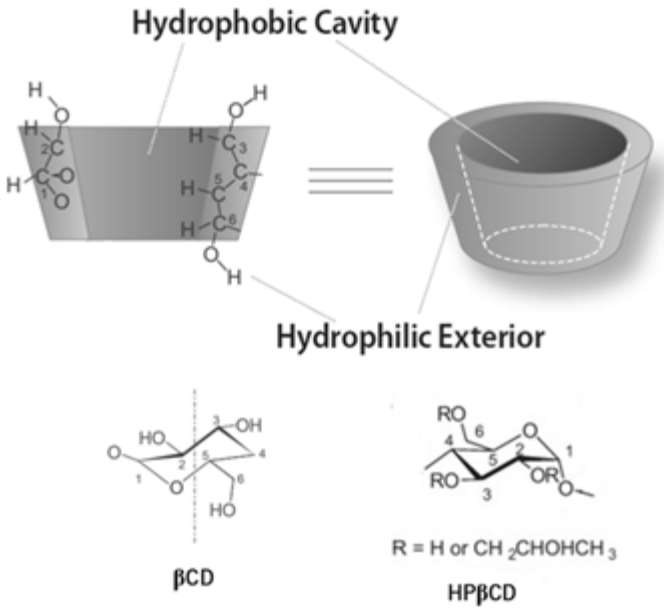

Fig 4.2.8 Position of the hydroxyl groups on the truncate shape of each cyclodextrin.

Therefore, the gel-β was formed by the interaction of βCD' OH-6 and the epoxy group of BDGE, resulting in a network with lower mobility (Fig 4.2.7). In the case of gel-HPβ (Fig 4.2.7), CD may be linked to the polymeric structure by the OH-6 and/or by the OH on the hydroxylpropyl groups, providing more flexibility to the polymeric network and, consequently, more viscoelastic and greater swelling.

Biological Properties of gel-β and gel-HPβ

Wound dressing are expected to be biocompatible and, therefore, suitable for contact with injury skin without causing any harm to the user. The hydrogels surface stated the interaction between the wound dressing and the skin. Those interactions can be predicted by the angle formed between the hydrogels surface and water or other solvents [24].

Hydrogels polymeric networks implies the ability of these materials to absorb large amounts of water, so it should be expected to obtained low contact angles, due to hydrogel hydrophilic nature [25]. According to the swelling results (Chaper 4.1), gel-HPβ should have low contact angle than the gel-β, but the opposite was observed. The contact angle value reflects the degree of freedom of the molecules network to move, as response to the environment change [25]. Therefore, the gel-HPβ showed reduced the mobility of their macromolecules, decreasing their surface hydrophilicity (Chapter 4.1). Moreover, the enhanced of gel-β hydrophilic surface after the caffeic acid loading can be a result of interactions between the caffeic acid and water. At pH 5, the caffeic acid is charged, and able to interact with the water molecules [26]. Also, on the IC, the caffeic acid carboxylic group (charge group) is project out of the βCD cavity, establishing interactions with the water molecules (Chapter 3.2). Thus, the gel-β surface hydrophilicity was increased by the interaction of caffeic acid with water.

Gel-β and gel-HPβ were able to sustain the caffeic acid release for 2 days with similar profiles (Fig 4.2.4). An initial burst was detected, as result of the transference of the caffeic acid present in the network aqueous phase to the solution. The remained delivery was slower, since it depends on the dissociation rate between the CDs and the phenolic. At equilibrium, the gel-HPβ allows higher release of caffeic acid, almost twice the amount observed for gel-β. At this point 2 processes are involved on the controlled release by CD-based hydrogels: (1) the swelling degree and (2) inclusion complex stability. The gel-HPβ had higher swelling capacity which enhanced the

caffeic acid mobility through the network. Additionally, the low gel-β release was a result of hampered caffeic acid dissociation from the CD cavity, since the complex formed with this CD was more stable than HPβCD (Chapter 3.2). This suggests that the sustained release was dependent on the CD ability to retain the caffeic acid in the network, as previously reported for cyclodextrin-based hydrogels load with hydrophobic molecules [3, 15, 20–22].

The antibacterial properties of the produced hydrogels are directly linked to the materials ability to deliver the caffeic acid. Based on the caffeic acid release results (0.77 mg. $g_{hydrogel}^{-1}$ for gel-β and 1.2 mg. $g_{hydrogel}^{-1}$ for gel-HPβ), the concentration of caffeic acid in contact with bacteria was, at least, 77 or 120 mM. Gel-β and gel-HPβ were able to induce meaningful reduction on the bacteria growth regardless the bacteria (Fig 4.2.5)

The antibacterial activity of caffeic acid appears to be deeply linked with the phenolic ability to reach the bacteria surface [27]. Thus, the caffeic acid loading into the polymeric networks allowed the caffeic acid interaction with electrons on the bacteria surface leading to cascade of events. That interaction induced a change on bacterial electric potential and on the cytoplasmic pH. The proton donation causes destabilization and disruption the cell membrane and, also, hyperacidification of cytoplasm [27]. Important enzymatic pathways can be disrupt leading to decrease of bacteria growth or even total inhibition [12, 28].

As stated above, hydrogels for wound dressing applications have to be friendly to the injury tissue, thus their cytotoxicity evaluation is crucial. Preliminary cytotoxicity assays of gel-β and gel-HPβ were performed based on the membrane integrity of the fibroblast cell, by MTS assay. This method has been often use for *in vitro* cytotoxicity evaluation of polymeric materials, and correlates the mitochondrial status with cell proliferation [2]. The caffeic acid effect on 3T3 fibroblast was described as dose-dependent, being safety for concentrations under 7mM (Chapter 2). Gel-HPβ allows only, 40% of the cells to survive as reflect of its higher ability to deliver caffeic acid (120 mM after 48 h). However, gel-β showed reasonable interactions with fibroblasts (viable cells above 70%), though the amount of caffeic acid release was 10 times the limit described for free phenolic compound. Accordingly, the caffeic acid loading into gel-β, apparently, reduced its cytotoxicity (Fig 4.2.6).

In conclusion, caffeic acid was successfully loaded into the CD-based hydrogels, and its release was kept for 2 days. Moreover, the loaded gel-β and gel-HPβ were capable of destroy most of the bacteria cells preserving the caffeic acid antibacterial activity. Nevertheless, gel-β appeared to be

more suitable for healing wounds, since it was friendly to fibroblasts. Thus, the developed cellulosic CD-based hydrogels, specially the gel-β, have a great potential as efficient carrier of caffeic acid, to be used as wound dressing.

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Chapter 5

Final Remarks and Future Perspectives

Final Remarks

The deposition and growth of bacteria on injury skin leads to infections, which increase the production of exudate, maceration of the tissue, and delay on the healing process. Hydrogels, (polymeric networks with the ability to swell after immersion in a liquid) have been, successfully, used as wound dressing, since they can retain the moist environment and absorb the exudate. The syntheses of hydrogels with cyclodextrins (CDs) allow the improvement of drug delivery ability of these networks. Then, CDs can form complexes with a wide range of molecules, altering their physiochemical and biological properties.

Nowadays, the demand for natural and friendly products has increased, as well as the source of new antibacterial agents (as consequence of the decrease of wide-spectra antibiotics activity against multi-resistant bacteria). Plants offer an immensity of bioactive molecules, being the poliphenolics one of the most studied groups. These molecules have been described as anti-inflammatory, anti-oxidant, bactericidal and fungicidal. However, polyphenolics applicability as therapeutics agents has been restricted by their lower stability to environmental factors, solubility in water or biofluids, and bioavailability. Moreover, cyclodextrins are known to enhance the solubility of polyphenolics in aqueous systems, protecting them from elevated temperatures, pH alterations, light or the moisture-induced degradations phenomena, and leading to the improvement of their bioavailability. β-cyclodextrin' (βCD) derivatives allow the formation of more stable inclusion complexes (IC) with flavonoids, when compared with native βCD. In the case of non-flavonoids, the three-dimensional fit between the bioactive molecules and the cavity of the cyclodextrins played a major rule on the inclusion complex stability. Nevertheless, CD-based hydrogels combined with polyphenolics gathers suitable properties for the wound healing. Thus, the development of a wound dressing with these features was the aim of this study.

First of all, a screening of antibacterial potential of phenolic extracts from wild Northeast Portuguese plants, as well as some of their phenolic compounds was made. From the extracts selected, the ones collected from *Cistus ladanifer, Castanea sativa, Filipendula ulmaria* and *Rosa micrantha* were effective in controlling the growth of bacteria commonly isolated from skin and soft tissue infections, in concentrations between 0.625 and 2.5 $mg.mL^{-1}$. Moreover, the *Filipendula ulmaria* and *Rosa micrantha* extracts can be safely used as antibacterial agents without causing any harm to the human cells. From the above extracts, 6 phenolic compounds

were selected: 3 flavonoids (kaempferol, quercetin and rutin) and 3 phenolic acids (ellagic, caffeic and gallic acids). Although, the flavonoids showed a growth inhibition halo against the gram positive bacteria, they were not active for concentrations below 5 $mg.mL^{-1}$. The phenolic acids were capable of control and inhibit the growth of 3 bacteria, with the exception of the ellagic acid. Thus, the caffeic and gallic acids were selected for the further work, as result of their antibacterial activity at reduced concentration. Regarding the effect on fibroblast growth and proliferation, caffeic acid had dose-response cytotoxicity, and can be used securely in concentrations below 6.31 $mg.mL^{-1}$. Gallic acid promoted the proliferation of fibroblasts for the lower concentration, but for concentrations above 0.1 $mg.mL^{-1}$ it became toxic. In the same range of concentration, the gallic acid was more toxic than the caffeic acid, probably because gallic acid is more acid (lower pKa). Therefore, Chapter 2 results reinforced the potential of phenolic compounds from North Eastern Portugal plants as antibacterial agents, for the treatment of infected wounds, instead of large-spectrum antibiotics. Based on the antibacterial and cytotoxicity results, the gallic and caffeic acid were chosen for incorporation on wound dressings, to avoid tissue infections.

As referred above, phenolic acids (as others polyphenolics) are susceptible to environmental conditions, such as pH and solvent. The effect of both factors on gallic acid UV-Vis spectra was analysed. Alterations on the gallic acid spectra were observed, the buffer modified the intensity of the peaks, and the pH interfered with the peaks position. Moreover, gallic acid became unstable at pH 8, as result of autoxidation. Additionally, gallic acid antibacterial properties were, also, affected by the pH, became less efficient at acid pH. Thus, the neutral form of gallic acid had lower ability to interact with bacteria.

In order to improve the gallic and caffeic acids' stabilization and solubilisation, their encapsulation by βCD and derivatives was studied. For the first phenolic, pH 3 was the most suitable condition for the formation of inclusion complexes with the 3 cyclodextrins, and all had a 1:1 stoichiometry. However, the stability parameters varied with the CD used, increasing in the following order methyl-β-cyclodextrin (MβCD), βCD and hydroxypropyl-β-cyclodextrin (HPβCD). The last 2 CDs were selected, based on higher stability parameters, for the antibacterial assessment of inclusion complexes activity. The gallic acid was still capable to reach the bacteria surface, and to inhibit their growth despite of being inside the CD cavity. In the case of caffeic acid, the pH 5 was the most suitable condition for the inclusion complex formation with βCD and

HPβCD. The complexes obtained, also, had 1:1 stoichiometry regardless the CDs, but βCD complexes had higher stability parameters. The encapsulation of caffeic acid by the βCD and HPβCD improved the phenolic antibacterial properties, probably caused by the improvement of caffeic acid solubilisation and reduction of aggregates formation.

Nevertheless, HPβCD/gallic acid and βCD/caffeic acid show the most suitable properties for their utilization as antibacterial agent, to be applied on the treatment of skin and soft tissue infections.

Since gallic and caffeic acids are effective against bacteria, commonly isolated from infected wounds, and their encapsulation for cyclodextrins enhanced phenolics properties, CD-based hydrogels were prepared to be applied as vehicles for the drug delivery of both phenolic acids, with favourable properties for the prevention and/or treatment of wound infections. The βCD and HPβCD were cross-linked with hydroxypropylmethyl cellulose (HPMC), using 1 4-butanediol diglycidyl ether (BDGE), since these CDs were able to form stable IC with both acids. The hydrogels showed suitable mechanical characteristics for contact with injured skin, such as viscoelasticity, smooth surface and superabsorbance. A mechanism of reaction for the hydrogel formation was proposed based on the analysis of swelling, DSC, FTIR and contact angle results. It was postulated that, the epoxy groups from BDGE react with the hydroxyl groups (OH) from the CDs and HPMC. In the case of gel-β, the OH-6 present on the narrower side of the CD had higher reactivity and, because of that, this was probably the group intervener on the reaction. The OH groups of the hydroxypropyl from the HPβCD substitutions had similar reactive to the OH-6 of βCD. Thus, this CD may be linked to the polymeric structure by the OH-6 and/or by the OH on the hydroxypropyl groups, providing more flexibility to the polymeric network and, consequently, more viscoelasticity and greater swelling.

Regarding the phenolics, the hydrogels were capable of successfully load the gallic and caffeic acids, and their controlled release occur for 48 h. However, it was observed that the load and release, of these phenolic acids, were intimately related to the CD within the network. Hence, the gel-HPβ was the network with best properties for the incorporation of gallic acid, and the gel-β was more suitable for caffeic acid. Nevertheless, the results obtained suggested that gallic and caffeic acids may be inside the cyclodextrins cavity and, also, trapped in the polymeric network. Regarding the biological properties of the hydrogels, the gallic and caffeic acid antibacterial ability was preserved after incorporation, as well as their interaction with fibroblast.

It is important to highlight that, to best of authors' knowledge, the loading and release of gallic and caffeic acids (as antibacterial agents) into hydrophilic networks of CD, HPMC and BDGE (as cross-linking), and its release for control wound infections, have not been evaluated until now.

In conclusion, the present thesis point out the applicability of natural antibacterial agents for the prevention and/or control of infected wounds, which can be enhanced by their incorporation on hydrogels wound dressing. The polymeric networks were synthetized using natural compounds and were friendly to fibroblast proliferation. Thus, the developed cellulosic CD-based hydrogel have a great potential as efficient carrier of gallic and caffeic acid, and can be used as wound dressing.

Future Perspectives

The work within this thesis highlights the potential of natural molecules as antibacterial agents, especially phenolic acids, and their incorporation within CD-based hydrogels. However, new information regarding the interaction of the phenolic compounds with the bacteria and animal cells will facilitate the optimization of wound dressings, and improve their ability to delivery those agents.

The phenolic acid interaction with bacteria has been general described. Thus, new insights regarding mechanism of action will bring valuable information about the molecules and/or chemical reactions responsible for the antibacterial properties of these phenolics. It will, also, clarify if the mechanism responsible for destroying bacteria is related to the one observed for the interaction with animal cells.

Another interesting study might be the antibacterial activity of gallic and caffeic acid on poly-microbial populations and biofilms. Wounds, are, usually colonized by complex microbial communities comprising a wide range of bacteria species. Thus, the effect of gallic and caffeic acids on other strains of *K. pneumoniae, S. epidermis* and *S. aureus*, and other bacteria species isolated from infected wounds, for example *Escherichia coli*, *Pseudomonas aeruginosa* or *Proteus sp*, could clarify the mechanism involved on the phenolics action and enhance the applicability of those molecules as antibacterial agents. Moreover, wounds colonizing bacteria can establish and proliferate, giving rise to biofilm formation. Biofilms interfere with the human immune system, facilitating the establishment of further bacteria communities and inflammation, and delay the healing process. Additionally, biofilms show higher tolerance to antibiotics than planktonic cells. Therefore, the study of gallic and caffeic acid antibacterial activity on these structures should be an asset on the natural antibacterial agents field. The gallic and caffeic acid should be capable of control biofilm proliferation, since they are small molecules with improved ability to diffuse to the interior of the biofilm structure.

In the present work, it was demonstrated that the developed hydrogels had suitable properties for their use as antibacterial wound dressings. However, in order to their commercialization, hydrogels should be subject to more specific evaluation regarding the interaction with human cells. Hence, the hydrogels effect on epithelial and keratinocytes cells lines should be evaluated, as well as, their effect on skin tissue models. The interaction hydrogel-cells could be assessed by

gather information from survival, metabolic, genotoxicity and irritation assays. Moreover, the evaluation of the hydrogels antibacterial active on infected skin tissue should, also, brings interesting insights to the hydrogels-cells-bacteria interaction. Untimely, the hydrogels should be evaluated regarding their *in vivo* healing ability using animal models.

As referred during the present thesis, CD-based hydrogels had enhanced ability to load hydrophobic bioactive molecules. Thus, it might be interesting to evaluate the versatility of the polymeric networks developed, during this work, as platforms for the delivery of other molecules (such as growth factors, anti-oxidants or local analgesics) capable of improving the healing process, in order to amplify the applicability of this wound dressings.

Summarizing, the recognition of the specific interactions between the gallic or caffeic acids with bacteria and animal cells should bring valuable insights about the mechanism of action of those compounds, and help the optimization of the delivery platforms such as the hydrogels developed.